INTERNATIONAL SEARCH REPORT

Intern .nal Application No
PCT/NL 00/00406

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/54 C12N15/62 C12N15/82 C08B30/00 A01H5/00
A01H5/10 A23L1/0522

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C08B A01H A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEN L ET AL: "IMPROVED ADSORPTION TO STARCH OF A BETA-GALACTOSIDASE FUSION PROTEIN CONTAINING THE STARCH-BINDING DOMAIN FROM ASPERGILLUS GLUCOAMYLASE" BIOTECHNOLOGY PROGRESS, vol. 7, - 1991 page 225-229 XP002056940 ISSN: 8756-7938 cited in the application the whole document ---	19
X	WO 92 11376 A (AMYLOGENE HB) 9 July 1992 (1992-07-09) the whole document ---	24,25, 28,30, 31,33,34
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 January 2001	30/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Kania, T

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/NL 00/00406

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CA 2 061 443 A (VISSER RICHARD G F ;JACOBSEN EVERT (NL); FEENSTRA WILLEM J (NL)) 19 August 1993 (1993-08-19) the whole document	24,25, 28-36
X	WO 98 14601 A (EXSEED GENETICS L L C) 9 April 1998 (1998-04-09) the whole document	1-36
A	WO 98 16190 A (NOVONORDISK AS ;TSUCHIYA RIE (DK); FUGLSANG CLAUS CRONE (DK)) 23 April 1998 (1998-04-23) cited in the application the whole document	1-19
A	WO 99 15636 A (NOVONORDISK AS) 1 April 1999 (1999-04-01) the whole document	1-19

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr. nal Application No
PCT/NL 00/00406

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9211376 A	09-07-1992	SE 467358 B	06-07-1992
		AU 9114891 A	22-07-1992
		CA 2098171 A	21-06-1992
		EP 0563189 A	06-10-1993
		EP 0788735 A	13-08-1997
		EP 0921191 A	09-06-1999
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		KR 210352 B	15-07-1999
		NO 932227 A	11-08-1993
		PL 169848 B	30-09-1996
		US 5824798 A	20-10-1998
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		EP 0935665 A	18-08-1999
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		EP 1001736 A	24-05-2000
WO 9915636 A	01-04-1999	AU 9254198 A	12-04-1999
		EP 1023438 A	02-08-2000

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 07 March 2001 (07.03.01)	
International application No. PCT/NL00/00406	**Applicant's or agent's file reference** BO 42696
International filing date (day/month/year) 13 June 2000 (13.06.00)	**Priority date** (day/month/year) 11 June 1999 (11.06.99)
Applicant VISSER, Richard, Gerardus, Franciscus et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

10 January 2001 (10.01.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Olivia TEFY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PA . _NT COOPERATION TREAT .

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

VEREENIGDE
Attn: Mr. J. Renes
Snouckaertlaan 42
N-3811 MB Amersfoort
PAYS-BAS

Date of mailing (day/month/year)
23 October 2001 (23.10.01)

Applicant's or agent's file reference
BO 42696

IMPORTANT NOTIFICATION

International application No.
PCT/NL00/00406

International filing date (day/month/year)
13 June 2000 (13.06.00)

1. The following indications appeared on record concerning:

☐ the applicant ☐ the inventor ☒ the agent ☐ the common representative

Name and Address

JORRITSMA, Ruurd
Nederlandsch Octrooibureau
Scheveningseweg 82
P.O. Box 29720
NL-2502 LS The Hague
Netherlands

State of Nationality | State of Residence

Telephone No.

Facsimile No.

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☒ the person ☐ the name ☐ the address ☐ the nationality ☐ the residence

Name and Address

VEREENIGDE
Attn: Mr. J. Renes
Snouckaertlaan 42
N-3811 MB Amersfoort
Netherlands

State of Nationality | State of Residence

Telephone No.

Facsimile No.

Teleprinter No.

3. Further observations, if necessary:
Please furnish telephone and fax numbers of the new agent.

4. A copy of this notification has been sent to:

☒ the receiving Office
☐ the International Searching Authority
☐ the International Preliminary Examining Authority
☐ the designated Offices concerned
☒ the elected Offices concerned
☐ other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Anman QIU

Telephone No.: (41-22) 338.83.38

P/ \T COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
BO 42696		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/NL 00/00406	13/06/2000	11/06/1999
Applicant		
LANDBOUWUNIVERSITEIT WAGENINGEN		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/[redacted]00/00406

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/54 C12N15/62 C12N15/82 C08B30/00 A01H5/00
A01H5/10 A23L1/0522

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C08B A01H A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 92 11376 A (AMYLOGENE HB) 9 July 1992 (1992-07-09) the whole document ---	24,25, 28,30, 31,33,34
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 January 2001	30/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

4

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/[redacted]00/00406

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	CA 2 061 443 A (VISSER RICHARD G F ;JACOBSEN EVERT (NL); FEENSTRA WILLEM J (NL)) 19 August 1993 (1993-08-19) the whole document	24,25, 28-36
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A	WO 99 15636 A (NOVONORDISK AS) 1 April 1999 (1999-04-01) the whole document	1-19

4

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/[redacted]00/00406

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9211376	A	09-07-1992	SE	467358 B	06-07-1992
			AU	9114891 A	22-07-1992
			CA	2098171 A	21-06-1992
			EP	0563189 A	06-10-1993
			EP	0788735 A	13-08-1997
			EP	0921191 A	09-06-1999
			FI	932804 A	17-06-1993
			HU	66754 A	28-12-1994
			JP	6507064 T	11-08-1994
			KR	210352 B	15-07-1999
			NO	932227 A	11-08-1993
			PL	169848 B	30-09-1996
			US	5824798 A	20-10-1998
CA 2061443	A	19-08-1993	NONE		
WO 9814601	A	09-04-1998	AU	4803097 A	24-04-1998
			BR	9713242 A	18-01-2000
			CN	1239514 A	22-12-1999
			EP	0935665 A	18-08-1999
			US	6107060 A	22-08-2000
WO 9816190	A	23-04-1998	AU	4550897 A	11-05-1998
			EP	1001736 A	24-05-2000
WO 9915636	A	01-04-1999	AU	9254198 A	12-04-1999
			EP	1023438 A	02-08-2000

Consommation et Corporations Canada
Consumer and Corporate Affairs Canada

Bureau des brevets
Patent Office

Ottawa, Canada
K1A 0C9

(21) (A1) 2,061,443
(22) 1992/02/18
(43) 1993/08/19

(51) INTL.CL.[5] C12N-005/10; A01H-005/00; A01H-005/06

(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Potato Plant Producing Essentially Amylose-Free Starch

(72) Visser, Richard G. F. - Netherlands ;
Jacobsen, Evert - Netherlands ;
Feenstra, Willem J. - Netherlands ;

(73) Same as inventor

(57) 12 Claims

Canada

ABSTRACT

Title: Potato plant producing essentially amylose-free starch

A potato plant which has a genome containing, as a result of genetic engineering, at least one gene construct containing a potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA sequence in reverse or functional orientation in an expression cassette which is functional in potato plants, said gene construct giving rise to tubers containing essentially amylose-free starch. In one embodiment, said gene construct contains a PGBSS cDNA sequence in reverse orientation which results in the production of PGBSS antisense RNA. In another embodiment, said gene construct contains a PGBSS genomic DNA sequence in functional orientation which results in co-suppression of PGBSS enzyme activity.

Title: Potato plant producing essentially amylose-free starch

Field of the invention

The invention is in the field of genetic engineering by recombinant DNA technology, more particularly the genetic engineering of potato plants in order to change the starch composition in the tubers towards essentially amylose-free starch.

Background of the invention

Starch is the major storage carbohydrate in potato and consists of two components, a linear (1→4)α-D-glucan polymer and a branched (1→4)(1→6)α-D-glucan called amylose and amylopectin, respectively. Amylose has a helical conformation with a molecular weight of 10^4-10^5. Amylopectin consists of short chains of α-D-glucopyranose units primarily linked by (1→4)α bonds with (1→6)α branches and with a molecular weight up to 10^7. In plants starch is found in two types of plastids: chloroplasts and amyloplasts. In both types of organelles the starch occurs as granules. In chloroplasts so-called transitory starch is accumulated for only a short period of time, whereas starch in amyloplasts is accumulated for long term storage and hence is named reserve starch. Generally, amylose makes up 11%-37% of the total reserve starch and variation in the amylose content is not only found among different plant species, but also among different cultivars of the same species. In potato the amylose content in the tuber varies from 18% to 23%. Furthermore, in a

number of plant species mutants are known with a starch composition which deviate significantly from the above mentioned percentages.

Transitory and reserve starch are generally considered to be synthesized by the same enzymes. Starch metabolism in leaves follows a diurnal rhythm: synthesis and accumulation occur during the light period while hydrolysis occurs during the night. In storage tissue, starch synthesis occurs during a specific phase of tissue development; the synthesis being the predominant function of amyloplasts. The amount of amylose found in storage tissue of potato is about twice as high as that in leaves.

Sucrose is considered to be the major substrate for starch biosynthesis which involves the following steps: initiation, elongation, branching and granule formation. In the pathway of conversion of sucrose into amylose and amylopectin at least 13 enzymes play a role. Three groups of enzymes are directly involved in the formation of starch. These enzymes are phosphorylase, starch synthases and branching enzymes. Phosphorylase is active in starch breakdown, branching enzyme converts amylose into amylopectin by the breakage of (1→4)α-bonds and the synthesis of (1→6)α-bonds. Starch synthases are responsible for the synthesis of starch by the addition of ADP (UDP) glucose subunits to the non-reducing end of an (1→4)α-D-glucan polymer. Starch synthase has been identified in two forms: one form is soluble while the other is tightly associated with starch granules. The soluble enzyme uses only ADP-glucose as the D-glucosyl donor, whereas the granule bound starch

synthase (GBSS) utilizes ADP-glucose and UDP-glucose. Solubilization of the GBSS protein from starch granules of various plants has been reported. Although in maize there are thought to be at least two forms of GBSS, potato seems to have only one form. The presence and activities of the different starch synthases are important to starch biosynthesis not only because they have an effect on the amylose/amylopectin ratio in starch, but also because they can have a large impact on total starch content. In general, it appears that complete suppression of the enzymes producing amylose can be achieved with almost no change in the total amount of starch laid down, whereas suppression of the enzyme system producing amylopectin leads to a marked decrease of the amount of total starch.

Starch as such or in modified form is widely used in the food, paper and textile industries. With the depletion of natural oil resources starch could also become a substitute for oil as a raw material for the chemical industry. Therefore, it could become of major interest to produce starch which meets special requirements for certain applications. Although special forms of starch are already available from mutants of maize and rice and starches from other sources might have certain advantages, genetical engineering could be an option in order to obtain tailor-made starches in plants in which (recessive) mutants are not easily obtained. Selection of mutants is especially difficult in vegetatively propagated crops which are mainly crosspollinators and/or polyploids, such as the potato.

Although recently in a laborious isolation procedure a mutant of potato (amf) was isolated which, in analogy to the wx

mutants in maize, lacks GBSS protein, GBSS activity and amylose (Hovenkamp-Hermelink et al. 1987), the breeding of such a mutant into a cultivar will take another number of years. One cause for the long duration of the procedure is the fact that a haploid clone had to be used for the isolation of the recessive mutant. To circumvent problems of isolating recessively inherited mutants in a polyploid crop like potato and to speed up the introduction of such a mutant character in potato varieties, the antisense approach would be a very important alternative, because an antisense gene would act as a dominant suppressor gene. The great advantage is that eventually it will become possible to mimick such a mutant phenotype directly in a tetraploid variety. With the availability of GBSS sequences, both from maize (Shure et al 1983) and potato (Hergersberg 1988; Visser et al 1989d) and an efficient transformation system for potato (Visser et al 1989a, 1989b) this approach could be tested.

It has been shown that antisense RNA transcripts can be used to mimic mutations in pro- and eukaryotes (for review see van der Krol et al. 1989). Antisense RNA was originally found as a naturally occurring mechanism used to control gene expression in bacteria (Tomizawa et al. 1981; Mizuno et al. 1984). Izant and Weintraub (1984, 1985) proposed that antisense RNA could be used to inhibit the expression of eukaryotic genes. By inhibiting the expression of specific target RNAs, this approach has led to the generation of mutant phenotypes in a number of different eukaryotic systems. In plants the use of antisense RNA proved to be successful in effectively inhibiting the activity of nopaline

synthase (Rothstein et al. 1987; Sandler et al. 1988), chloramphenicol acetyltransferase (Ecker and Davis 1986; Delauney et al. 1988), chalcone synthase (van der Krol et al. 1988), polygalacturonase (Smith et al. 1988; Sheehy et al. 1988), phosphinotricin acetyl transferase (Cornelissen and Van de Wiele 1989) and β-glucuronidase (Robert et al. 1989).

Visser (1989) tested whether the antisense approach could be used to inhibit the expression of the gene for granule-bound starch synthase in potato using heterologous antisense constructs, i.e. an antisense gene constructed from a maize genomic GBSS gene.

The antisense gene was fused between the 35S cauliflower mosaic virus promoter and the nopaline synthase terminator in the binary vector pPOK-1, which also carries a plant selectable kanamycin resistance gene. Since it was known from the amf-mutant that the mutation is expressed in all tissues in which starch is formed, including columella cells of the root cap, it was expected that also antisense effects would be visible in roots. The presence or absence of amylose could be easily detected because amylose forms a blue staining complex with the iodine present in Lugol's solution (I-KI). Starch without amylose, i.e. only containing amylopectine, forms a reddish-brown staining complex with iodine. In order to efficiently test the introduced antisense gene in potato for a biological effect a transformation system was developed in which the binary antisense vector was incorporated into Agrobacterium rhizogenes. The binary vector was present next to the wildtype Ri-plasmid of A. rhizogenes which is responsible for the formation of so-called hairy roots on plant

explants. Agrobacterium rhizogenes was used inst ad of Agrobacterium tumefaciens because it is possible to screen for an effect of the introduced constructs already after 10 days by staining hairy roots with Lugol's solution and because plants can be easily regenerated from hairy roots. In this way heterologous (maize) binary antisense GBSS plasmids were transferred by A. rhizogenes to stem segments from potato.

Both in untransformed or otherwise transformed wildtype roottips never anything else than blue staining rooctips were present. Hairy roots obtained after transformation with A. rhizogenes carrying heterologous binary antisense GBSS plasmids were analyzed for the presence or absence of amylose in their starch by staining the roottips with Lugol's solution. The majority of the roots stained blue as wildtype untransformed roots did. However, some roots (1-15% of the stained roots) had a colour pattern different from that of wildtype roots in that the central cells of the root cap were blue and the cells towards the outside of the rootcap were red. These intermediate colouring roots were indications that the inserted antisense genes had some effect on the amylose content. Root clones were established and subcultured and roottips were investigated every fortnight during six weeks of culture. The results of these experiments showed that instability of colour patterns occurred at a rather high frequency. The instability of the effect in columella cells was the reason to regenerate plants from kanamycin resistant hairy roots irrespective of their colour.

On plants, regenerated from kanamycin resistant hairy roots, microtubers as well as soil grown tubers were induced. Analysis

of these tubers showed that none of them had red or intermediate staining starch. All tubers showed blue staining (= amylose containing) starch. Starch isolated from these tubers was analyzed for the presence of GBSS protein and GBSS activity and for the presence of amylose. In all tubers tested GBSS protein was, seemingly unaltered, present. However, GBSS activity in particular and to a much lesser degree amylose content were affected in starch preparations from a number of transformed plants. As shown in fig. 2A, the untransformed wildtype (PD007) and a pBI121 transformed wildtype (Ri-007) had similar GBSS activities, while the amf-mutant had no detectable GBSS activity. GBSS activity was inhibited significantly in the antisense GBSS transformants down to only 10% of that found in wildtype plants. Total inhibition of GBSS activity was not obtained in any of the transformants analyzed. The amylose content measurements gave a different picture. Although in almost all cases there was a somewhat lower amylose content, the difference was significant in only two cases R-196 and R-227, Figure 2B). The maximum reduction of the amylose content was found in transformant R-196, which also had the lowest GBSS-activity; a reduction down to 78% of the wildtype amylose content. Molecular analyses of the antisense transformants revealed that the number of integrated antisense copies was 1 to 4, but only those plants which contained three or more copies of the antisense GBSS construct showed a pronounced effect on GBSS activity. It is evident from these observations that the effect of a lower GBSS activity on the amylose/amylopectin ratio is not straightforward.

The results described resemble very closely the situation obtained in tomato when using antisense poly-galacturonase genes. A reduction of 90% of the polygalacturonase activity does not have a great effect on the lycopene content (Sheehy et al. 1988, Smith et al. 1988).

The above results were not too encouraging, but it was nevertheless decided to expand the investigations to homologous constructs derived from a full-length potato GBSS cDNA.

Surprisingly, it was found that it is possible to inhibit the expression of granule-bound starch synthase (GBSS) in potato, and thus affect the amylose content of potato tuber starch, by stably introducing homologous antisense constructs. The results described show that it is possible using the antisense approach to interfere with enzymes in biosynthetic pathways such as starch biosynthesis. In using this technique loss of function mutations, such as the amf mutation, which are principally inherited recessively can be mimicked, because antisense genes act as dominant (hemizygous) genes suppressing translation of mRNA.

Surprisingly, it was subsequently found that the effect of essentially amylose-free tuber starch could also be obtained by stably introducing homologous sense constructs, e.g. based on potato GBSS genomic DNA. A phenomenon known as co-suppression appears to occur; it is not yet possible to give an explanation of it.

Summary of the invention

The invention provides a potato plant which has a genome containing, as a result of genetic engineering, at least one gene construct containing a potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA sequence in reverse or functional orientation in an expression cassette which is functional in potato plants, said gene construct giving rise to tubers containing essentially amylose-free starch.

In one preferred embodiment, said gene construct contains a PGBSS cDNA sequence in reverse orientation which results in the production of PGBSS antisense RNA.

In another preferred embodiment, said gene construct contains a PGBSS genomic DNA sequence in functional orientation which results in co-suppression of PGBSS enzyme activity.

The invention further provides cells, parts and tubers of said potato plant, and essentially amylose-free starch from it.

Detailed description of the invention

The invention will be illustrated by means of examples which are given for illustrative purposes only and may not be construed as limiting the scope of the invention. For example, the transformation system used in example 1 (Agrobacterium rhizogenes) may be replaced by any suitable alternative, such as the Agrobacterium tumefaciens transformation system (see ex. 2) or the direct gene transfer technique (DGT). Such alternatives are well known to the person skilled in the art. A survey of transformation systems suitable for potato is given in chapter I of Visser (1989).

Similar remarks apply to the choice of the transformation vector (if any), the elements of the expression cassette, the selection markers, etc. For example, the PGBSS promoter may be used to regulate the transcription of the sense or anti-sense PGBSS DNA, instead of the CaMV promoter used in example 1. The sense or anti-sense PGBSS cDNA or genomic DNA sequence does not have to cover the complete coding sequence but should cover a sufficient part of it to be effective for obtaining tubers containing essentially amylose-free starch. At present, the use of anti-sense PGBSS cDNA is preferred above using anti-sense PGBSS genomic DNA. The gene construct used may contain the PGBSS DNA (preferably genomic DNA) in its functional orientation and yet result in essentially amylose-free tuber starch.

Description of the Drawings

Fig. 1 shows the construction of the sense and antisense granule-bound starch synthase (GBSS) vectors. The original GBSS cDNA which contained an internal EcoRI site was subcloned as two fragments in pUC9, denoted pWx 1.1 and pWx 1.3. The 1.3 kb GBSS cDNA fragment from pWx 1.3 was ligated into the partial EcoRI-restricted plasmid pWx 1.1 yielding pGB2. Plasmid pGB2 was restricted with SpeI, made blunt ended with Klenow enzyme, BamHI linked and restricted with BamHI. The GBSS cDNA fragment was ligated into BamHI-restricted pUC18 yielding pGB6 and into BamHI-digested calf intestinal phosphatase (CIP) treated pROK-1 yielding pGB50 (antisense) and pGB60 (sense). Abbreviations: B, BamHI; E, EcoRI; H, HindIII; S, SpeI; LB, RB, left and right T-DNA border repeats; Km^r, kanamycin resistance marker expressed

at bacterial level; NPT-II (neomycin phosphotransferase II gene) kanamycin resistance marker expressed at plant level; P_{CaMV}, 35S cauliflower mosaic virus promoter; T_{NOS}, nopaline synthase terminator.

Fig. 2 compares the antisense effects on GBSS activity and amylose content of tuber starches in the case of heterologous constructs (2A, 2B) and homologous constructs (2C, 2D).

2A. GBSS activities of control (PD007, Ri-007 and amf) potato and antisense transformed potato tuber starches.

GBSS activity of wildtype PD007: 86.2 pMol ADP glucose min^{-1} mg $starch^{-1}$

GBSS activity of mutant amf: 1.3 pMol ADP glucose min^{-1} mg $starch^{-1}$.

2B. Apparent amylose content of control potato and antisense transformed potato tuber starches

wildtype PD007: 18.4%

mutant amf : 0%

2C. GBSS activity of control (PD007, Ri-007 and amf) potato and class I (WA 501, WA 511), class II (WA 504) and class III (WA 507, WA 514) antisense transformed tuber starches.

wildtype PD007: 66.9 pMol ADP glucose min^{-1} mg $starch^{-1}$

mutant amf : 0 pMol ADP glucose min^{-1} mg $starch^{-1}$

2D. Apparent amylose content of control and antisense transformed potato tuber starches

wildtype PD007: 20.3%

mutant amf : 0%

EXAMPLE 1

Materials and methods

Plant materials

In vitro shoot cultures of the Solanum tuberosum clones PD007 (HH 578, 2n=2x=24), R1J07 (pBI121 transformed PD007, 2n=2x=24; Visser et al. 1989a, b) and the doubled amf mutant (2n=2x=24; Hovenkamp-Hermelink et al. 1987) were used. The plants were grown at 21°C with a regime of 16 h light (3200 lux) per day on basal MS medium (Murashige and Skoog 1962) supplemented with 30 g l sucrose.

Standard methods and reagents

Standard techniques of DNA manipulation were performed as described by Maniatis et al. (1982). All DNA-mediated transformations were carried out with Escherichia coli strains JM83 (Vieira and Messing 1982) and MH1 (Casadaban and Cohen 1980). Plasmid isolation was according to Birnboim and Doly (1979) and inserts were isolated from the restriction enzyme-digested plasmids using the "freeze-squeeze" method (Tautz and Rer. 1983). Plant DNA was isolated according to Dellaporta et al. 1983). DNA blot hybridizations and radioactive labelling of the isolated 1.3 kb GBSS cDNA fragment from pGB6 (Fig. 1) were as described previously (Visser et al. 1989 b,c,d). Enzymes required for the DNA constructions were from Gibco-BRL and or Boehringer Mannheim and were used according to the manufacturers' recommendations.

Construction of vectors

Two subclones encompassing a full-length cDNA clone from potato GBSS isolated from a lambda NM1149 library (Hergersberg 1988; Visser et al. 1989d) were used as indicated in Fig. 1 for the construction of the antisense and sense binary vectors. The antisense (pGB50) and sense (pGB60) vectors obtained after the ligation of the 2.3 kb cDNA in the alkaline phosphatase-treated BamHI site of the binary plant transformation vector pROK-1 (Baulcombe et al. 1986) were introduced into Agrobacterium rhizogenes LBA 1334 (Offringa et al. 1986) as described by Visser et al. (1989a). Verification of the integrity of the plasmids in Agrobacterium was as described previously (Visser et al. 1989a).

Iodine staining of hairy roots

Starch granules in root cap cells were stained with a 1:1 (v/v) mixture of Lugol and chloral hydrate as previously described by Jacobsen et al. (1989).

Transformation of potato

Inoculum preparation of Agrobacterium cells carrying the antisense or sense constructs, inoculations of stem segments of S. tuberosum PD007 and isolation of binary vector-transformed hairy roots were as described (Visser et al. 1989a). Callus induction on hairy roots, followed by the subsequent regeneration of shoots was as described previously (Visser et al. 1989a). Plants grown in vitro were transferred to the greenhouse to obtain soil-grown tubers.

Assays for amylose content and GBSS activity

The amylose/amylopectin ratio in starch from potato tubers was determined by the method described previously (Hovenkamp-Hermelink et al. 1988). By using the equation postulated there: P=(3.5-5.1 R)/(10.4 R-19.9) where R is the ratio of the absorbance at 618 nm and 550 nm, the amylose fraction (P) can be easily determined. Since, when P was calculated for artificial mixtures of amylose and amylopectin of known composition, slight deviations were found between the calculated ratio and the actual ratio, a correction factor was used based on the ratio of calculated and actual values of P in these artificial mixtures. Stock solutions of amylose and amylopectin were made as described by Hovenkamp-Hermelink et al. (1988), mixed to obtain starch solutions containing 0% to 40% amylose and diluted with water to obtain final starch concentrations of 6.25 mg/100 ml.

GBSS activity was measured in 50 µl assay buffer containing 1.0-2.0 mg of enzymatically active starch granules using ^{14}C-labelled ADP-glucose as substrate, as described by Vos-Scheperkeuter et al. (1986). Suspensions were incubated for 30 min and 90 min at 37°C to get a linear incorporation of ^{14}C-ADP-glucose.

Gel electrophoresis and immunoblotting

Proteins were extracted from starches as described by Vos-Scheperkeuter et al. (1986). Analysis on 10% SDS polyacrylamide gels and subsequent immunoblotting or silver staining of proteins were performed as described by Vos-Scheperkeuter et al. (1986) and Hovenkamp-Hermelink et al. (1987).

Results

Transformation, hairy root isolation and shoot regeneration

Wild-type strains of A. rhizogenes and those harbouring the antisense (pGB50) and sense (pGB60) DNA constructs of GBSS (Fig. 1) were used to inoculate stem segments of wild-type S. tuberosum PD007. A. rhizogenes was used because columella cells of root tips contain starch and because the mutation in GBSS leads to the absence of amylose in columella cells in the amf mutant. Since the presence of amylose can be easily monitored by staining hairy roots with iodine (starch staining blue when amylose is present and reddish-brown when amylose is absent), this provides an easy screening method.

Hairy roots, when induced and grown on kanamycin-free medium were obtained in about 10 days after inoculation. Numerous root tips of untransformed PD007 shoots and hairy roots obtained on PD007 stem segments after inoculation with wild-type and pGB60 A. rhizogenes strains were tested for their starch composition in columella cells. These preparations always contained blue staining starch when stained with Lugol's solution. Roots inoculated with pGB50 could be classified into three staining groups, i.e. blue, intermediate and reddish-brown. In the intermediate class blue and red staining columella cells were found within the same root tip. Over 50% of the pGB50 inoculated roots showed an altered starch composition, staining either intermediate or red with iodine (Table 1).

To study intra-clone variation 10 root clones, which were established on kanamycin-containing medium, were investigated for starch composition every fortnight during 6-weeks (Table 2).

It was found that the antisense effect occurred at a high frequency, but mostly in an unstable fashion. Young root clones with red staining amylose-free starch in the columella cells tended to change into intermediate ones and at a later time after inoculation changed again into amylose-containing, homogenously blue staining root clones. Although one red root clone appeared to be stably amylose-free, the majority of the root clones was unstable for this antisense effect. All these observations clearly demonstrated that antisense effects for this character could be obtained in columella cells of root tips of transformed potato, but that suppression of amylose synthesis is unstable in such root tips.

Because of the instability problems it was decided to regenerate plants, irrespective of the antisense effect in their columella cells, from kanamycin-resistant hairy roots containing vector T-DNA in order to investigate these effects in other starch-containing parts of transgenic plants. Hairy roots transformed with pGB50 were isolated from stem segments and cultured on medium containing 50 mg/l kanamycin and 200 mg/l claforan. After two more rounds of subculturing, hairy root clones which still grew on kanamycin-containing medium were considered transformed. Each independently derived hairy root was subcultured separately on MS 30 medium with claforan and kanamycin to increase root mass. Root pieces obtained from 46 independently transformed PD007 hairy root clones (designated WA 500 to WA 546) were transferred to callus induction medium. All root clones formed callus, and shoot regeneration was observed in 25 (=54%) of the WA clones. Of these 16 were analysed

further; after in vitro multiplication 5 plants from each of these 16 clones, as well as control plants (pBI121 transformed PD007 and untransformed PD007 plants), were transferred to the greenhouse and soil-grown tubers were harvested.

Tuber starch analysis

From all 16 clones subterranean tubers were harvested. Two to three randomly picked tubers from every plant were cut in slices and the cut surfaces were stained with Lugol's solution. Based on the staining reaction three classes were discerned. Eleven plants formed tubers with red staining (amylose-free) starch and three plants formed tubers with only blue staining (amylose-containing) starch, whereas two formed tubers with a mixed staining type of starch. The mixed staining tubers were different from the intermediate staining root tips in that individual cells, which were grouped in a certain zone of the tuber, contained either red or blue staining starch. All tubers from such plants were of mixed staining phenotype. The sizes of the blue and red zones varied. Always the heel side of the tuber (the side attached to the stolon) contained blue staining starch.

Analysis of isolated starch from tubers of the three different staining classes revealed that activity and/or amount of GBSS protein were affected in all the different plants transformed with antisense constructs. Table 3 shows that all plants staining red for tuber starch, which were investigated for those characters, had strongly decreased GBSS activities comparable to that of the amf mutant, whereas all plants with

only blue staining tuber starch had significant GBSS activities, which, however, were lower than that of the wild type PD007. The GBSS activity in pBI121 transformed Ri007 plants was equal to that of the wild type. The amylose/amylopectin ratio was determined in tuber starch and the apparent percentage of amylose was calculated. The relative amylose content of tubers with blue staining starch was in some transgenic plants comparable to that of the wild type, PD007 or Ri007, while in other plants it was much lower. In tubers with red staining starch the amylose level of the amf mutant was achieved. The mixed staining tubers had amylose percentages which varied between those seen for the tubers with red and blue staining starch respectively, mainly because these tubers consisted of a mixture of amylose-free and amylose-containing starch.

The results are also shown in fig. 2C and 2D to facilitate a comparison with the heterologous transformants. As can be seen in Figure 2C, two out of the three different classes of tubers from homologous transformants contained GBSS activities which correspond with their colour when stained with Lugol's solution. Levels similar to that of the amf-mutant for red staining tubers and detectable but clearly lower than wildtype GBSS activities for blue staining tubers. The amylose content for these two classes is also in agreement with the colours of the tubers (fig. 2D). No amylose for the red staining tubers and rather high amylose contents comparable to those found in wildtype tubers for the blue staining tubers. The latter group also closely resembles the heterologous transformants. Starches isolated from the 'intermediate' colouring class gave a

completely different result. An example of such a transformant is WA504, which has a higher GBSS activity than both transformants from the 'blue colouring' class, WA507 and WA514, but the apparent amylose content of this transformant is much lower than that of the other two transformants.

GBSS protein analyses were performed by running protein extracts from a number of tuber starch samples on SDS polyacrylamide gels followed by subsequent silver staining of the gels or by transferring them onto nitrocellulose filters and immunoblotting with antibodies raised against GBSS from potato. From both silver stained gels or from the immunoblots it is evident that only blue and intermediate staining tubers contain GBSS protein in their starch granules. Although the extraction of protein from starch is difficult to quantify it seems that in the blue staining tubers little reduction of the level of GBSS protein has occurred. As demonstated by the weaker signal on the immunoblot for clones WA 504 and WA 517, a reduced level of GBSS protein is clearly found in the intermediate staining tubers.

Molecular analysis of the transformants

Southern blot analyses were performed to determine the number of correctly introduced antisense GBSS constructs in the transgenic plants. Genomic DNA from a number of independently obtained transformants was isolated and digested with EcoRI and BglII. These restriction endonucleases excise the construct in such a way that the number of different bands corresponds to the number of different integrations. In this way it was found that the number of integrations in the independent transformants

varied from 1 to 5. In untransformed PD007 or pBI121 transformed plants only the hybridization pattern from the resident GBSS genes could be discerned. No relationship was found between the antisense effects and the number of integrated copies. Both plants with tubers with red or blue staining starch could contain either one or more than one antisense copy.

Stability of expression of the antisense genotype

The antisense GBSS gene is expressed and the antisense-derived phenotype is visible, as is the amf phenotype, in all tissues where starch is formed; apart from columella cells of root tips and tubers this also includes stomatal guard cells. However, although the composition of the starch in the tuber of a given plant seemed to be quite stable, there was always a variable expression of the antisense genotype in stomatal guard cells and in columella cells. Root tips and guard cells with red and with blue staining starch could be found in one and the same plant, irrespective of its tuber starch reaction. The only variability in tuber starch colour was found in the transformants with mixed staining starch. These always had a varying zone of blue and red staining starch in their tubers; no variegated patterns or differently oriented zones were ever observed in the mixed staining tubers. Vegetative propagation of tubers with red staining starch from three transformants showed that the antisense trait is transmitted after multiplication and thus is apparently stably integrated into the genome.

Discussion

In this example we describe effects of the introduction of GBSS antisense constructs into potato on the amount and activity of GBSS and on the amylose/amylopectin ratio in potato starch.

Analysis of the hairy roots revealed that the introduction of pGB50 antisense constructs resulted in phenotypic changes, which did not occur when pGB60 sense constructs were used, as judged by the staining of starch in columella cells with Lugol's solution. However, because of the instability of the observed effect in columella cells this system is only suitable for testing constructs for their potential biological effects. Therefore, plants were regenerated from kanamycin-resistant roots irrespective of the root tip phenotype with respect to starch composition. In contrast to the hairy root clones investigated which were mostly blue staining, all but three plants formed tubers with stably red (11 plants) staining starch. None of the investigated transgenic plants, not even the blue ones, had a GBSS activity which was comparable to that of the wild type controls. In this respect the antisense approach was successful in all kanamycin-resistant plants.

The fact that variable expression of the antisense genotype occurs in stomatal guard cells, in which transitory or metabolic starch is deposited, as well as in columella cells of root tips is difficult to explain. Since such metabolic starch, at least in leaves, contains less amylose than reserve starch (Hovenkamp-Hermelink et al. 1988), it might be expected that an effect could be accomplished more easily in leaves. Since less amylose and less GBSS mRNA (Visser et al. 1989) are present a

suppressing effect in leaves would be more likely and would also be more stable. If the starch in roottips were also of metabolic origin identical results would be expected. However, our results contradict this expectation. In the guard cells of leaves the results obtained could point to the involvement of a physiological component such as photosynthesis, in roots such an involvement is more difficult to imagine. Another reason for the differences between effects obtained in tubers and in other organs of the plant can perhaps be found in the more constant expression of GBSS mRNA in tubers. The promoter used in this study could also be the reason for the observed phenomenon. The 35S CaMV promoter is considered a constitutive promoter in all tissues, but reports from Benfey and Chua (1989) and Benfey et al. (1989) have shown that this need not always be true. There are at least two domains in the promoter region (Benfey et al. 1989) which, when introduced separately into transgenic plants, can confer different developmental and tissue-specific expression patterns. In our case the complete 35S CaMV promoter perhaps allows specific expression levels in various tissues at different times during the day like those reported for the two different domains of the 35S CaMV promoter (Benfey et al. 1989). More research in the field of variable expression is necessary before one can hope to explain the observed results.

Here, however, we can only draw conclusions from the results derived from tuber starch analysis, since only in tuber starch does the expression appear to be stable and is measurable quantitatively. The data show a reduction of GBSS activity in all the investigated transgenic plants. In those cases where

there is total absence of GBSS activity, GBSS protein and amylose are also absent. In all other cases there is an inhibitory effect on activity and possibly also on the amount of GBSS protein. The effect of a lower GBSS activity on the amylose/amylopectin ratio is not straightforward, as was also seen in heterologous GBSS antisense experiments (Visser 1989). The strong reduction in GBSS activity required to evoke only a rather small reduction in the amylose/amylopectin ratio in transformants WA 507 and WA 514 are in contrast to that found in transformant WA 516, which has a higher GBSS activity but a lower amylose content. The interpretation of the effect on amylose/amylopectin ratio is complicated further by the fact that no data on absolute amounts of starch are available.

The introduction of antisense GBSS constructs clearly has an effect on a number of related parameters in starch metabolism. The fact that there is variation among different plants transformed with the same antisense construct is not surprising. Similarly variable responses of plants to particular antisense constructs have also been obtained for antisense chalcone synthase (CHS) in Petunia hybrida and Nicotiana tabacum (van der Krol et al. 1988), antisense chloramphenicol acetyl transferase in tobacco (Delauney et al. 1988) and antisense polygalacturonase (PG) in tomato (Smith et al. 1988). Moreover, in the animal systems variable inhibition of the target genes by antisense vectors has also been observed (Holt et al. 1986).

It is assumed that the variations in response arise from differential influence on antisense expression of the chromosomal region in which it is integrated, i.e. so-called

position effects (Van der Krol et al. 1988; Smith et al. 1988). All GBSS antisense transformed plants contained variable numbers of integrated antisense constructs, but no relationship between GBSS copy number and the observed antisense effect could be found. These results are similar to those observed by Van der Krol et al. (1988), where there was no correlation between antisense CHS copy number, antisense CHS mRNA level and phenotypic effects on flower pigmentation. These results differ from those obtained using heterologous GBSS constructs in potato (Visser 1989) where a correlation was found between GBSS copy number and phenotypic effect. A relationship between copy number and antisense effect was also found for antisense polygalacturonase genes in tomato (Schuch et al. 1989) and for antisense chloramphenicol acetyl transferase (CAT) in constitutively expressing chloramphenicol acetyltransferase (CAT^+) tobacco plants (Delauney et al. 1988).

In conclusion, this example shows that the introduction of GBSS antisense cDNA constructs results in a strongly reduced GBSS activity, which in most cases is accompanied by an equally large reduction in the amount of GBSS protein and amylose content. However, in those cases where the antisense effect is not absolute the degrees of reduction in the amount of GBSS activity, GBSS protein and the amylose content are unpredictable. The antisense approach can be a very effective alternative technique to mutagenesis programmes for enzymes involves in the metabolic pathway of starch formation, especially in vegetatively propagated (polyploid) crops. Moreover, the availability of antisense plants should make

normally recessively inherited mutations more easily obtainable, because antisense genes themselves act as dominant (hemizygous) suppressor genes, thus enabling plant breeders to shorten their breeding programmes.

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TABLE 1

Iodine staining of starch in columella cells of hairy roots formed on stem segments of Solanum tuberosum PD007 after inoculation with Agrobacterium rhizogenes pGB50.

Experiment	No. of roots stained	Percentages of roots staining		
		Blue	Intermediate	Red
I	43	40	21	39
II	49	41	21	38
III	103	34	33	34
IV	34	41	18	41
V	78	33	31	36
Total	319	38	24	38

Table 2

Iodine staining behaviour of starch in columella cells of kanamycin-resistant hairy roots containing pGB50 and analysed over a period of 42 days.

Root clone	Days after oculation			
	1	15	30	42
1	R——>	R——>	R——>	R
2	R——>	R* I——>	B——>	I B
3	I——>	R* I B——>	I* B——>	I B
4	I——>	I* B——>	B——>	B
5	B——>	B——>	B——>	B
6	R——>	I——>	R* I——>	I
7	R——>	R——>	R* I B——>	I B
8	I——>	R* I——>	I* B——>	I B
9	I——>	B——>	I* B——>	I B
10	B——>	B——>	B——>	B

R. red staining; I, intermediate staining; B, blue staining.

For every measurement obtained 3-8 root tips were stained. Of root clones with different staining root tips the clones marked with an asterisk were transferred to fresh medium.

Table 3

Tuber starch colour, GBSS activity and amylose content of antisense GBSS transformed potato plants and their controls.

Plant clone	Tuber starch colour after iodine staining	GBSS activity (as % of the content of PD007)	Apparent amylose content (as % of the content of PD007)
Class I			
WA 501	red	0	0
WA 505	red	3	2
WA 506	red	0	2
WA 508	red	0	2
WA 511	red	0	0
WA 512	red	2	3
Class II			
WA 504	blue/red	18	26
WA 517	blue/red	6	22
Class III			
WA 507	blue	9	83
WA 514	blue	10	84
WA 516	blue	32	49
Controls			
PD007	blue	100	100
Ri007	blue	96	106
amf	red	0	0

The tuber starch colour was determined by staining a cross-section of a tuber with Lugol's solution as described in the Materials and methods. GBSS activities measured as the incorporation rate of ^{14}C-labelled ADP-Glucose, ranged from 0 to 8.1 pmol/min per mg starch in individual tubers with red staining starch, from 8.7 to 70 pmol/min per mg starch in individual tubers with blue staining starch and from 6.2 to 32.7 pmol/min per mg starch in individual tubers with mixed staining starch. The values of untransformed or pBI121 transformed PD007 ranged from 66.9 to 98.9 pmol/min per mg starch and for the amf mutant were approx. 0 pmol/min per mg starch. Amylose percentages varied from 0% to 1.9% for tubers with red staining starch, from 14% to 27% for tubers with blue staining

starch and from 5% to 7% for tubers with mixed staining starch. Transgenic plant clones WA 502, WA 503, WA 509, WA 513 and WA 515, all containing red staining tuber starch, were regarded as class I transformants but were not included in the above analysis.

EXAMPLE 2

This example investigates if it is possible to increase the amylose content in potato tubers. This might be achieved by overexpressing granule-bound starch synthase if this enzyme is rate limiting to amylose biosynthesis. In order to investigate this question a full length genomic, actively transcribed (Visser et al. 1989, van der Leij et al. 1991), GBSS gene was introduced in a number of different wildtype and heterozygous (Amfamf) potato genotypes. The results of these experiments show that no significant increase of the amylose content could be obtained in any of the transformants. Surprisingly it was found that the introduction of this gene can instead block the expression of both the introduced and endogenous GBSS genes.

Materials and methods

Plant materials

In vitro shoots of the Solanum tuberosum genotype cv. Astarte (2n=4x=48), PD 007 (HH 578, 2n=2x=24), the Amfamf genotype 871024-2 (2n=2x=24) and the amfamf genotype 871029-31 (2n=2x=24, Jacobsen et al. 1989) were used. The shoots were grown at 21°C with a regime of 14h light per day on basal MS medium (Murashige and Skoog 1962) supplemented with 30 g/l sucrose (MS 30).

Standard methods and reagents

Standard techniques of DNA manipulation were performed as described by Maniatis et al. (1982). Plasmid DNA isolations from Escherichia coli were according to Birnboim and Doly (1979) and inserts were isolated from the restriction enzyme digested plasmid using the method described by Tautz and Renz (1983). Enzymes were from Gibco/BRL or Boehringer Mannheim and were used according to the manufacturers recommendations.

Construction of vectors and transformation of potato

The construction of the binary plasmid pWAM 100 containing a full length GBSS genomic gene, capable of complementing an amylose-free mutant, was described before (van der Leij et al. 1991). The binary plasmid was introduced into Agrobacterium rhizogenes LBA 1334 using triparental mating (Visser et al. 1991b). The binary vector pWAM 100 was also introduced into Agrobacterium tumefaciens LBA 4404 using the direct transformation method of competent Agrobacterium cells described

by Höfgen and Willmitzer (1988). Integrity of the plasmids in Agrobacteria was verified according to Holmes and Quigly (1981).

Potato stem segments were inoculated with A. rhizogenes as described by Visser et al. (1989a). The formation of hairy roots was allowed to take place on solid MS 30 medium with 200 mg/l cefotaxim, without kanamycin. Regeneration of shoots from hairy roots was as described before (Visser et al. 1989a). Transformation of potato stem segments with A. tumefaciens followed by subsequent regeneration of shoots from these explants was as described by Visser (1991).

Microtubers were obtained by transferring nodal buds to MS medium containing high amounts of sucrose (80 g/l) as described by Hovenkamp-Hermelink et al. (1987).

Plants grown in vitro were transferred to the greenhouse to obtain soil-grown tubers.

Screening of tubers

Subterranean tubers obtained from A. rhizogenes or A. tumefaciens transformants (respectively R-n or T-n) were cut and the surface was stained with Lugols solution.

Assays for GBSS activity and amylose content

GBSS activity was measured in 50 µl assay buffer containg samples of 1-2 mg of enzymatically active starch as described by Vos-Scheperkeuter et al. (1986) using ^{14}C labelled ADP-glucose as substrate.

The amylose/amylopectin ratio in starch from potato tubers was determined by the method described previously (Hovenkamp-Hermelink et al. 1988).

Gel electrophoresis and immunoblotting

Proteins were extracted from starches as described by Vos-Scheperkeuter et al. (1986). Analysis on 10 % SDS polyacrylamide gels and subsequent immunoblotting or protein staining were performed as described by Vos-Scheperkeuter et al. (1986) and Hovenkamp-Hermelink et al. (1987).

Southern and northern analysis

DNA of greenhouse grown plants was isolated from young leaves according to Dellaporta et al. (1983) and digested with the restriction enzymes EcoRI and BglII. Southern blot hybridizations with radioactive labelled GBSS cDNA was performed as described previously (Visser et al. 1989b,d).

RNA isolation from tubers and leaves, followed by northern blotting and hybridization were as described (Visser et al. 1989d).

Results

The diploid genotype PD007 and the tetraploid cv. Astarte were used in transformation experiments using the Agrobacterium rhizogenes strain harbouring the binary vector pWAM100.

The diploid genotype 871024-2 was used in transformation experiments harbouring the vector pWAM100. Table 1 shows the

number of individual transformants obtained using either transformation approach.

The transformants were allowed to tuberize in the greenhouse. All A. tumefaciens transformants produced tubers whereas only 65% of the A. rhizogenes transformants produced tubers. From all tubers starch was isolated to determine amylose/amylopectin ratio and from most of the tuber starch samples also the GBSS activity was measured (Table 5).

From the results of Table 5 it is obvious that none of the transformants had a significantly higher amylose content as compared to the wildtype controls. However, both in the A. rhizogenes and the A. tumefaciens transformants clones were found containing red staining starch in their tubers. Also in the case of the tetraploid cv Astarte where 12 transformants produced tubers (Table 4), one clone was found with red staining sectors in the tubers (results not shown). This was also the case in one of the 871024-2 transformants (T-21). In all transformants with red staining tuber starch the GBSS activity proved to be very low or undetectable. In these particular transformants no detectable amounts of amylose could be measured (Table 5, clones R-11, R-30a and T-40), thus resembling the amf-mutant for these characters.

The phenotype from these transformants in other starch containing tissues was different from that of the amf-mutant; stomata and roottips had blue and only occasionally red staining starch. In this respect these transformants resembled more some anti-sense GBSS transformed clones (Visser et al. 1991a).

These results were due to the fact that transformants contained extra copies of the GBSS gene, as was confirmed both by polymerase chain reaction experiments as well as by Southern blot analysis. The number of integrated copies varied from one to four per haploid genome and no relation between copy number and inhibitory effect was found (results not shown).

Discussion

In this example, the effect of the introduction of additional homologous GBSS gene copies in diploid (both heterozygous and homozygous for the amylose-free character, respectively Amfamf and AmfAmf) and tetraploid potatoes on GBSS activity and amylose percentage is investigated.

Although it was the intention to increase expression of the GBSS gene leading to an increased activity of the enzyme, something which was reported for sense alfalfa glutamine synthetase in transgenic tobacco (Eckes et al. 1989) no such phenomenon was observed in our experiments. Unexpectedly the introduction of extra copies of the GBBS gene led to a (almost) complete inhibition of the GBSS enzyme activity in 8% to 22% of the transformants (Table 5), irrespective of their ploidy level or genetic constitution for the amf-character which in the heterozygous diploid means that only one copy of the gene has to be suppressed to achieve an effect. The lower or absent GBSS activity was accompanied with lower amounts or total absence of GBSS protein when analysed by Western blot (cf. Table 5). The absence of GBSS protein and activity in turn led to an almost complete absence of amylose (<1% of the wildtype level) and

could be made visible by staining cut tuber surfaces with Iodine: presence of amylose gave blue staining, absence of amylose red staining starch (Table 5).

The phenomenon that a mutant phenotype can be obtained after introduction of one or more copies of a wildtype gene in a wildtype host has been described before (Napoli et al 1990, Smith et al. 1991) and is known as co-suppression. One common feature of co-suppression in different plant systems seems to be the occurrence of instable or reversible phenotypes. An indication for this phenomenon in the five sense GBSS transformants showing an effect in the tuber, might be the starch composition in other starch containing organs of these plants. Another example of such an unstable or reversible phenotype might be transformant T-21 which contained partly blue and red staining starch in its tuber. It is thought that these phenotypes are related to natural cases of gene expression like flower colour patterning, and epigenetic effects as in paramutations and other modulating mechanisms of transposition (Jorgensen 1991, Matzke and Matzke 1991).

At present two explanations for the feature of co-suppression are in favour: methylation or anti-sense RNA effects. Methylation is thought to be a result of interactions of homologous sequences at different sites in the genome. Several examples which share similarities with co-suppression are known to be related to methylation. In these cases homology between promoters seems to be essential and suppression acts at the level of transcription (Matzke et al. 1989, Matzke and Matzke 1991). In our case homology with the complete sequence

including the promoter region with resident sequences exists. However, none of the 12 potato clones transformed with a chimaeric gene consisting of the GBSS promoter and the β-glucuronidase (GUS) gene contained amylose-free starch (Visser et al. 1991b, unpublished results).

A second explanation for co-suppression involves the so called anti-sense RNA interaction. It was postulated by Grierson et al. (1991) that anti-sense RNA could be generated because of simple read through of the kanamycin resistance gene which is cotransferred for selection and used sofar in all the systems known to show the phenomenon of co-suppression (van der Krol et al. 1990, Napoli et al. 1990, Grierson et al. 1991, Matzke and Matzke 1991). However, the fact that the GBSS promoter, which was used in our case, is a much more powerful promoter than for instance the 35S (CaMV) promoter (Visser et al. 1991b) makes this unlikely. Rather the place of integration, also known as position effect, seems to play a more important role. It is known from experiments with promoterless constructs that most of the integrations take place in regions of the genome which are trancriptionally active (Koncz et al. 1989, Goldsbrough and Bevan 1991). In potato about 10% of the plants transformed with a promoterless GUS-gene showed GUS activity in tubers and/or leaves.

According to this example, additional copies of the structural gene granule-bound starch synthase (GBSS) were transferred into different potato genotypes using either Agrobacterium tumefaciens or Agrobacterium rhizogenes as a vector to investigate the possibility of increasing the amylose

content in potato tubers. Out of eighteen transformants, only two had a higher GBSS expression, but this did not lead to increased amounts of amylose in tuber starch. Surprisingly, however, in five transformants a strongly reduced GBSS activity was found. In the three transformants which showed the highest reduction in GBSS activity, this severe reduction was accompanied by the absence of GBSS protein and amylose in the starch granules. The specific inhibition of GBSS expression involved both the introduced and the endogenous gene. The effect obtained was not related to the ploidy level of the plant nor to the number of extra gene copies introduced into the plant.

In conclusion, this example shows that the introduction of an actively transcribed granule-bound starch synthase gene into potato may lead to suppression of the expression of both the introduced and the endogenous gene.

Table 4

The number of (tuberizing) transformants obtained either by using A. rhizogenes or A. tumefaciens harbouring the binary vector pWAM 100.

Plant genotype	Bacterium	No. of transformants	with tubers
PD007	R	19	9
Astarte	R	16	12
871024-2	T	30	30

R= A. rhizogenes, T=A. tumefaciens

Table 5

Comparison of GBSS activity, presence of GBSS protein, amylose content and starch colour of pWAM 100 transformants with the untransformed controls (PD007, 871024-2 and 871029-31).

Plant	GBSS activity as % of wt*	Amylose content as % of wt**	GBSS protein	Tuber starch colour
PD007 (AmfAmf)	100	100	+	blue
R-5	nd	84	+	blue
R-6	nd	86	+	blue
R-11	0	<1	-	red
R-19	nd	79	+	blue
R-20	nd	86	+	blue
R-24	129	102	+	blue
R-27	nd	73	+	blue
R-29	nd	95	+	blue
R-30a	4	0	-	red
871024-2 (Amfamf)	100	100	+	blue
T-4	18	75	+	blue
T-7	131	95	+	blue
T-11	49	75	+	blue
T-13	46	95	+	blue
T-21	10	30	±	red/blue
T-25	81	100	+	blue
T-26	56	95	+	blue
T-34	44	95	+	blue
T-40	3	0	-	red
881029-31 (amfamf)	0	0	-	red

nd = not determined

GBSS protein presence determined by Western blot analysis,

+ = present, - = absent

* PD007 and 87 1024-2 activity ranged from 65 to 100 pMol/min/mg starch, 871029-31 activity from 0 to 4 pmol/min/mg starch

** PD007 amylose % 18 to 27 %

871024-2 amylose % 18 to 24 %

871029-31 amylose % 0 to 4 %

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A potato plant which, as a result of genetic engineering, has a genome containing at least one gene construct containing a potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA sequence in reverse or functional orientation in an expression cassette which is functional in potato plants, said gene construct giving rise to tubers containing essentially amylose-free starch.

2. The potato plant of claim 1, wherein said gene construct contains a PGBSS cDNA sequence in reverse orientation which results in the production of PGBSS antisense RNA.

3. The potato plant of claim 1, wherein said gene construct contains a PGBSS genomic DNA sequence in functional orientation which results in co-suppression of PGBSS enzyme activity.

4. The potato plant of claim 1, wherein said expression cassette contains the cauliflower mosaic virus 35S promoter (P_{CaMV}).

5. The potato plant of claim 1, wherein said expression cassette contains the PGBSS promoter.

6. The potato plant of claim 1, wherein said expression cassette contains the nopaline synthase terminator (T_{nos}).

7. The potato plant of claim 1, wherein said expression cassette contains the PGBSS terminator.

8. The potato plant of claim 1, wherein said gene construct contains the neomycin phosphotransferase II gene (NPT-II) kanamycin resistance marker.

9. A cell of the potato plant of any of the claims 1-8.

10. A part of the potato plant of any of the claims 1-8.

11. A tuber of the potato plant of any of the claims 1-8.

12. Essentially amylose-free starch from the potato plant of any of the claims 1-8.

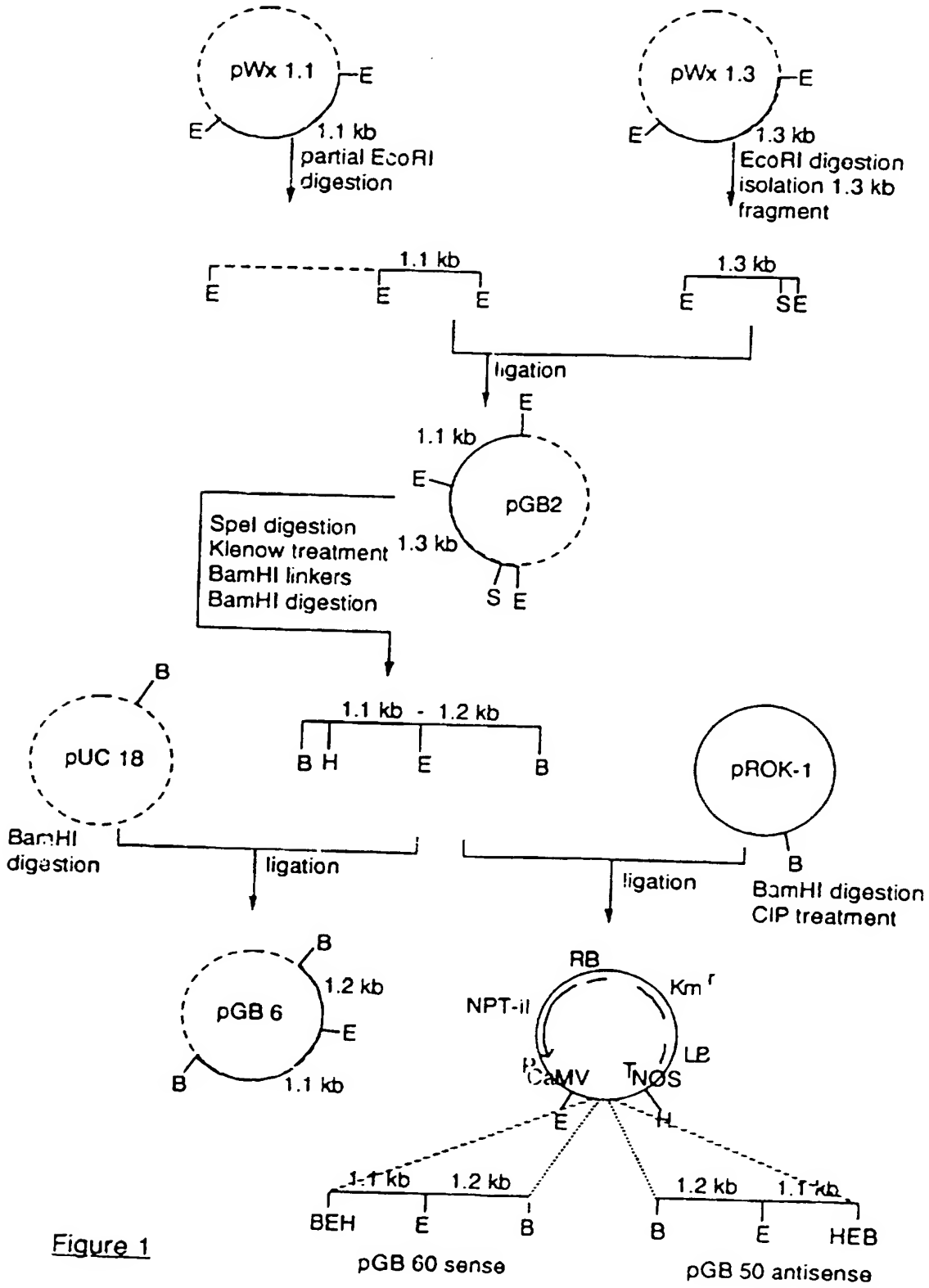

Figure 1


GBSS activity (% of PD007)
100
50
0
PD007
RI-007
amf-1
R-196
R-227
R-164
R-175
R-200

FIG. 2A

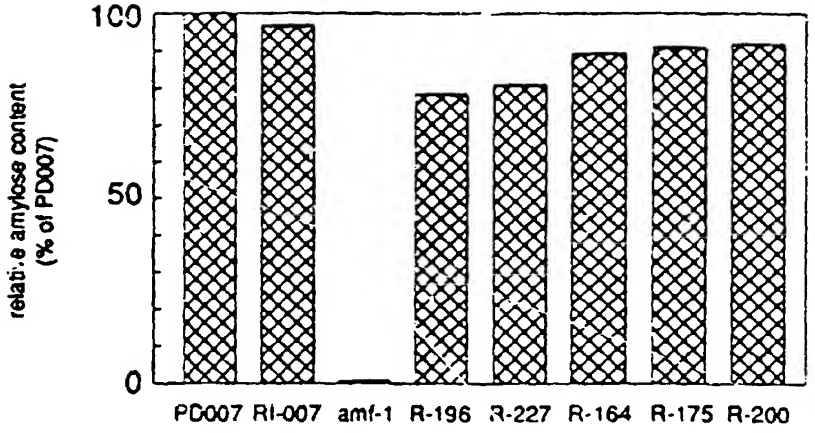
relati:e amylose content
(% of PD007)
100
50
0
PD007
RI-007
amf-1
R-196
R-227
R-164
R-175
R-200

FIG. 2B

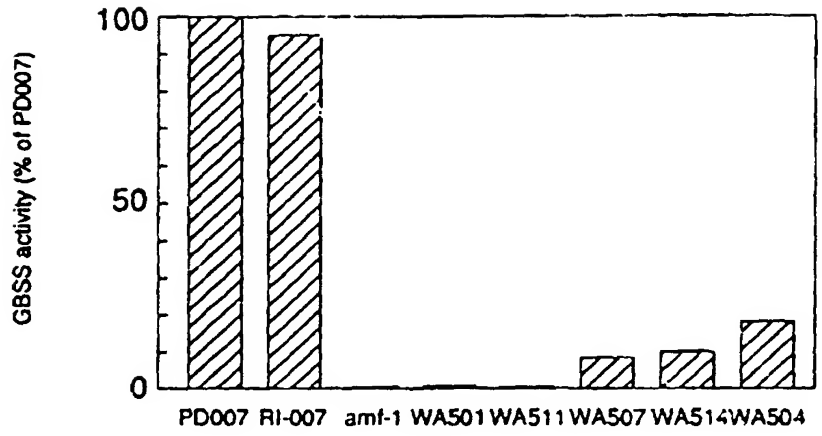
GBSS activity (% of PD007)
100
50
0
PD007 RI-007 amf-1 WA501 WA511 WA507 WA514 WA504

FIG. 2C

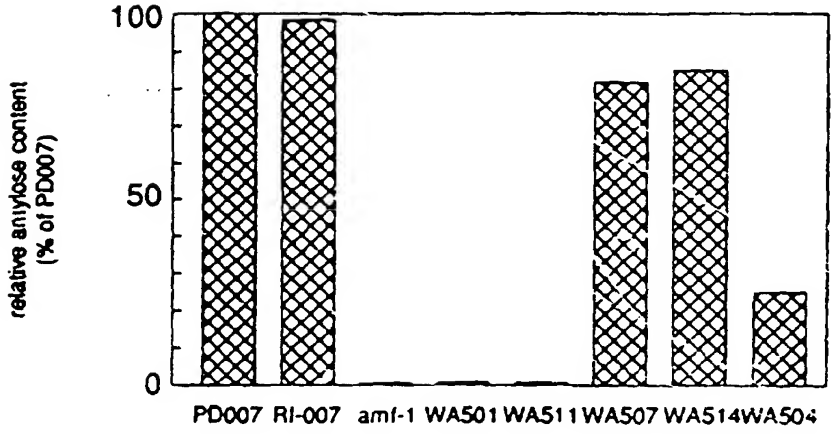
relative amylose content (% of PD007)
100
50
0
PD007 RI-007 amf-1 WA501 WA511 WA507 WA514 WA504

FIG. 2D

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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(54) Title: EXPRESSION IN PLANTS OF STARCH BINDING DOMAINS AND/OR OF PROTEIN FUSIONS CONTAINING STARCH BINDING DOMAINS

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INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/54 C12N15/62 C12N15/82 C08B30/00 A01H5/00
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEN L ET AL: "IMPROVED ADSORPTION TO STARCH OF A BETA-GALACTOSIDASE FUSION PROTEIN CONTAINING THE STARCH-BINDING DOMAIN FROM ASPERGILLUS GLUCOAMYLASE" BIOTECHNOLOGY PROGRESS, vol. 7, - 1991 page 225-229 XP002056940 ISSN: 8756-7938 cited in the application the whole document	19
X	WO 92 11376 A (AMYLOGENE HB) 9 July 1992 (1992-07-09) the whole document	24,25, 28,30, 31,33,34

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	CA 2 061 443 A (VISSER RICHARD G F ;JACOBSEN EVERT (NL); FEENSTRA WILLEM J (NL)) 19 August 1993 (1993-08-19) the whole document	24,25, 28-36
X	WO 98 14601 A (EXSEED GENETICS L L C) 9 April 1998 (1998-04-09) the whole document	1-36
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A	WO 99 15636 A (NOVONORDISK AS) 1 April 1999 (1999-04-01) the whole document	1-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

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(57) Abstract: The invention relates to a method for expressing a desired protein or polypeptide in a plant, in which the protein or polypeptide is expressed as a fusion with at least one starch binding domain. The plant is preferably a plant that contains or produces starch or starch granules in at least one of its parts, such as potato, sweet potato, cassava, pea, taro, sago, yam, banana and/or cereals such as rice, maize, wheat and barley. The protein or polypeptide can be an enzyme, in particular an enzyme that can convert, modify, alter, degrade or otherwise influence starch (granules); or can be a receptor or a structural protein. The invention further relates to the fusions thus obtained, to genetic constructs that encode the above fusions and to plants transformed with said constructs. The method of the invention can in particular be used to provide modified starches and/or to provide complexes of starch (granules) and the above fusions. In another embodiment, one or more starch binding domains are expressed in a plant, to provide a plant producing modified starches.

Expression in plants of starch binding domains and/or of protein fusions containing starch binding domains.

The present invention relates to methods for gene expression in plants.

In particular, the invention relates to the expression in plants of proteins and/or polypeptides - including enzymes - as fusions with improved affinity for starch, such as for the starch granules that may occur in plants *in vivo*, in particular in cellular organelles such as plastids

More in particular, the invention relates to the expression in plants of proteins and/or polypeptides as a fusion with one or more moieties or domains that have affinity for starch, such as 'starch binding domains'.

Some further aspects of the invention reside in the protein fusions thus expressed, in gene constructs that encode such fusions, and in methods for the transformation of plants using such constructs as well as in the transformed plants thus obtained.

The invention can in particular be used for the expression in plants *in vivo* of enzymes as fusions that become associated with the starch granules that occur in (some of the cellular organelles of) such economically important plants as potato, sweet potato, cassava, pea, taro, sago, yam banana, and/or cereals such as maize, rice wheat, and barley. This may either facilitate the isolation of the enzymes thus expressed from the plant material, as well as their further use, and/or the enzymes thus expressed may be used to alter and/or otherwise influence the starch or starch granules leading to plants with improved properties, such as the production of modified starches. These and other applications of the invention will become clear from the description hereinbelow.

International application WO 98/14601 describes a hybrid polypeptide comprising: (a) a starch binding domain, and (b) a payload polypeptide fused to said starch binding domain. Said hybrid polypeptide may be expressed in any suitable host organism, such as in bacteria, plants and animals.

Said starch binding domain - referred to in WO 98/14601 as "*starch-encapulating domain*" - may be any starch-binding domain known per se, for instance derived from soluble starch synthase I, soluble starch synthase II, soluble starch synthase III, granule-bound starch synthase, branching enzyme I, branching enzyme IIa, branching enzyme IIBb or glycoamylase.

The general purpose of the method described in WO 98/14601 is to provide a "*peptide-modified starch*" (vide for instance page 10, line 20) , i.e. the "*encapsulation of desired amino acids or peptides within the starch and specifically within the starch granule*" (page 6, line 28) to "*increase the plants capacity to produce a specific protein, peptide or provide an improved amino acid balance.*" (page 21, line 1).

For instance, the method of WO 98/14601 may for instance be used "*to make modified starches comprising the payload polypeptide*" (page 7, line 8); "*for providing polypeptide such as hormones or other medicaments, e.g. insulin, in a starch encapsulating form to resist degradation by stomach acids*" (page 7, line 11); "*for producing the payload polypeptides in easily purified form*" (page 7, line 13); or "*to enhance the amino acid content of particular amino acids in the modified starch*" (page 8, line 1) to provide "*grain feeds enriched in certain amino acids*" (page 7, line 9).

As specific examples of payload polypeptides, WO 98/14601 mentiones "*hormones, e.g., insulin, a growth factor, e.g. somatropin, an antibody, enzyme, immunoglobulin, or dye*" (page 8, lines 7-9), as well as for instance prolactin, serum albumins and growth hormones (page 15, lines 1-16).

As preferred payload polypeptides, WO 98/14601 mentiones somatotropin, insulin A and B chains, calcitonin, beta endorphin, urogastrone, beta globin, myoglobin, human growth hormone, angiotensin, proline, proteases, beta-galactosidase, and cellulases (pages 15, lines 14-16).

However, none of these payload proteins or polypeptides is capable of "interacting" with starch or the starch granules as defined in this application.

More generally, WO 98/14601 does not describe fusions of at least one starch binding domain and at least one "starch-altering" enzyme as described hereinbelow, i.e. an enzyme that can convert, modify, alter, degrade or otherwise influence the starch, the starch granule or the structure or interactions thereof.

WO 98/14601 also does not describe, nor mentions as a purpose or as a possible application, the expression of fusions of at least one starch binding domain and an enzyme that can "interact with" starch and/or a starch granule in a plant, e.g. to provide a transgenic plant that is capable of producing a modified starches or modified starch granules as described hereinbelow, i.e. a starch (granule) that differs from the starch (granule) naturally provided by the plant in at least one property thereof, such as crystallinity, branching degree, glucan composition, oxidation, phosphorylation, etc..

(In this respect, it should be noted that where WO 98/14601 refers to a "*modified starch*", a "*peptide-modified starch*" is meant, e.g. "*the naturally occuring starch* (that) *has been modified to comprise the payload polypeptide*", vide page 9, lines 27-28. Compared to the corresponding native starch, such a "(peptide-)modified" starch according to WO 98/14601 will only be "modified" compared to the starch natively produced by the plant in that it comprises said protein or polypeptide.).

The international application WO 92/11376 describes a method for suppressing amylose formation in potato by transforming a potato plant with a construct comprising antisense fragments designed to inhibit (the expression of) the GBSS-gene. The Canadian patent application 2 061 143 describes a similar technique for producing amylose-free potato starch.

In starch-producing plants, starch is usually synthesized/present in the form of starch granules. A number of enzymes in the plant are known to interact *in vivo* with these granules, for instance in order to build up, modify and/or degrade the starch molecules, the starch granules and/or the structure thereof. These include enzymes such as starch synthases, branching and debranching enzymes, etc., for which in general reference is made to A.M. Smith, K. Denyer and C. Martin, Annu. Rev. Plant Physiol. Mol. Biol.,1997, 48:67-87 and C.Martin and A.M. Smith, The Plant Cell, Vol.7, 971-985, July 1995.

Such enzymes or mutants thereof may also be used to produce modified starches, either *in vitro* or *in vivo*. The production of modified starches by plants tranformed with (genes encoding) such enzymes is for instance described in DE-A-195 34 759, WO 92/14827 (in which a branching enzyme derived from potato cDNA is used) and WO 92/11376 (which describes an alternative method in which antisense DNA is used to suppress GBSS activity in plants.)

It is also known that some micro-organisms contain proteins/enzymes that can interact with (i.e. degrade, modify or convert) starch or starch granules, and some non-limiting examples thereof are mentioned below. Again, such enzymes and/or mutants thereof have been used in the art to produce modified starches *in vitro* and/or *in vivo*, the latter in plants transformed with (a) gene(s) encoding said enzyme or mutant. Reference is for instance made to WO 91/19808, US-A-5,349,123 and US-A-5,750,875,which describe plants transformed with bacterial amylases - and in particular

cyclodextringlycosyltransferases (CGTases) - for the *in vivo* production of cyclodextrins.

Proteins/enzymes that can interact with starch, and in particular the enzymes from micro-organisms, generally contain - besides one or more catalytic domains - one or more regions that can bind to the starch and/or the starch granules. The latter are referred to in the art as "starch binding regions" or "starch binding domains". For a description of some starch binding domains that have been investigated in the art, reference is for instance made to Penninga et al., J. Biol. Chem, Vol.271, No 51, 32777-32784, 1996, and Lawson et al., J. Mol. Biol. (1994) 236, 590-600, who describe the raw starch binding domain (E-domain) of CGTase from *B.circulans*; Sorimachi et al., J. Mol. Biol. (1996), 259, 970-987 and Structure 1997, Vol.5, No.5, 647-661, who describe the starch binding domain of *A. niger* glucoamylase.

Svensson et al., Biochem. J. (1989), 264, 309-311, describe the sequence homology between putative starch binding domains from α-amylase from *Streptomyces limosus*, β-amylase from *Clostridium thermosulfurogenes*, glucoamylase from *A. niger*, maltogenic α-amylase from *Bacillus stearothermophilus*, maltotetraose-forming amylase from *Pseudomonas stutzeri*, CGTase from *Bacillus*, CGTase from *Klebsiella pneumoniae* and glucoamylase from *Rhizopus oryzae* (the latter being a N-terminal starch binding region). A similar comparison of different starch binding domains is also given by Janecek and Sivcek, FEBS letters 456 (1999), 119-125, published after the priority date of the present application.

It has also been suggested that some conserved tryptophan residues and the amino acids directly adjacent thereto may play an important role in starch binding, vide Goto et al., Appl. Environ. Microbiol., 1994, p.3926-3930; Williamson et al., Biochemistry 1997, 36, 7535-7539, and Chen et al., Protein Engineering, vol.8, 1049-1055 (1995).

WO 98/16190 describes fusion products of enzymes and one or more starch binding domains, as well as oral care compositions that contain such fusions. The fusions are prepared by expression of an appropriate expression vector in a suitable microorganism.

WO 99/15636 describes starch binding domains, and in particular the so-called "D-" and "E-domain" of the maltogenic amylase from *Bacillus stearothermophilus* C599, and expression thereof in a *Bacillus* host cell. WO 99/1536 also described

fusions of said starch binding domain and a reporter gene such as *gfp*, in order to monitor the expression of the starch binding domains in the *Bacillus* host.

However, WO 99/15636 does not describe fusions of a starch binding domain and an enzyme that can interact with starch or a starch granule. Also, WO 99/15636 only describes expression in *Bacillus*.

Chen et al., in Gene 99, (1991), 121-126, and in Biotechnol. Prog. 1991, 7, 225-229, describe a fusion of β-galactosidase and the starch binding domain from an *Aspergillus* glucoamylase, plasmids encoding such a fusion, and expression of said fusion in *E.coli*. The starch binding region is used to increase the affinity of β-galactosidase for starch granules, in particular as an affinity tail for recovery or enzymatic immobilisation using native starch granules as an absorbent.

Dalmia et al., in Biotechnology and bioengeneering, Vol.47, pp.575-584 (1995), describe fusions of β-galactosidase and the starch binding domains of glucoamylase I of *Aspergillus awamori* and of cyclodextrin glucanotransferase (domain E of CGTase) from *Bacillus macerans*, respectively, plasmids encoding said fusions, and expression of said fusions in *E.coli*. The fusion proteins thus obtained are said to bind specifically to potato starch, corn starch, and cross-linked amylose. As a possible application, the use of the starch binding domains as an "affinity tag" is suggested. Similarly, Dalmia et al., in Enzyme Microb. Technol.,1994, vol.16, describe fusions containing a starch binding domain from *A. niger* glucoamylase, which is again used as an affinity tail to facilitate the one-step purification of the target β-galactosidase.

The use of cellulose binding domains as an affinity tag for protein purification (i.c. a fusion of a cellulose binding domain from a cellulase and α-galactosidase) has also been described in the art, vide Ong et al., Trends in Biotechnology, 7, 239-243 (1989).

However, when the above references describe fusions of a starch binding domain and an enzyme, said fusions are expressed in a micro-organism such as *E.coli*. Also, the starch binding domain is included only as a "tail" or "tag" in order to facilitate the isolation and purification of the desired enzyme activity from the bacterial culture medium.

The expression and use of such fusions in plants *in situ*, in particular in association with starch granules and/or plastids, is not mentioned in the art. In particular, the expression in plants of fusions containing enzymes that can alter the

properties of the starch (granules) present in the plant (for instance for the *in vivo* production of improved starches) has not been described or suggested.

It is general object of the invention to express desired proteins or polypeptides in plants *in vivo* in association with starch produced or present in the plant, in particular in association with the starch granules produced or present in a plant.

A further object of the invention is to provide a method for obtaining transformed plants that produce modified starches.

Other objects of the invention will become clear from the description and figures hereinbelow.

The above objects are achieved by expressing a desired protein or polypeptide that can interact with starch or starch granules, in the plant as a fusion with one or more starch binding domains.

By "interact with starch or starch granules" is generally meant that said protein or polypeptide can convert, modify, alter, degrade or otherwise influence the starch, the starch granule or the structure - and in particular the fine structure - or any interaction(s) thereof, and/or the physical and/or chemical properties of said starch (granule). Generally, this will result in a starch or starch granule that differs from the starch (granule) naturally provided by the plant in at least one property thereof (i.e. in addition to the presence of the fusion), such as crystallinity, branching degree, glucan composition, oxidation, phosphorylation, etc..

Usually, said protein or polypeptide will be an enzyme that can interact with starch or starch granules, including but not limited to the enzymes mentioned hereinbelow. Such enzymes will also be referred to as "starch altering enzymes", and the fusions of such starch altering enzymes with one or more starch binding domains will also be referred to as "starch altering fusion".

Thus, compared to the teaching of WO 98/14601, such a "starch altering fusion" according to the invention is capable of "actively" influencing at least one property of the starch (granule), e.g. as indicated below. For this purpose, the "starch altering enzyme" will be an enzyme that natively (i.e. also when not expressed as a fusion as described herein) shows some affinity and/or (enzymatic) activity towards starch (granules). In particular, these will be enzymes that as such already can convert, modify, alter, degrade or otherwise influence starch, a starch granule or the structure or any interaction(s) or properties thereof. In particular, these will be enzymes that as such

can already alter at least one property of starch (granules), and in particular one or more of the properties 1-15 listed below.

By comparison, the proteins, polypeptides or enzymes that are mentioned for the fusions according to WO 98/14601 natively do not show any affinity and/or (enzymatic) activity towards starch (granules).

In its broadest sense, the invention relates to a method for expressing a desired protein or polypeptide in a plant, in which the protein or polypeptide is expressed as a fusion with at least one starch binding domain.

In particular, the invention relates to a method for expressing a desired protein or polypeptide in a plant, such that the desired protein or polypeptide becomes associated and/or can be obtained in association with any starch produced by or present in said plant, and in particular with any starch granules produced by or present in said plant or any part of said plant, in which the protein or polypeptide is expressed *in vivo* in the plant or any part thereof as a fusion with one or more starch binding domains.

More specifically, this method comprises the steps of:

a) providing a genetic construct comprising at least one nucleotide sequence encoding the desired protein or polypeptide combined with at least one nucleotide sequence encoding a starch binding domain;
b) transforming a plant with said genetic construct;
c) expressing said genetic construct in the plant *in vivo*.

Furthermore, as the starch granules in a plant will usually be present in cellular organelles within the plant cell, and in particular in plastids such as amyloplasts, chloroplasts and/or chromoplasts, the method of the invention can also be used to express a desired protein or polypeptide in association with such an organel.

In a further aspect, the invention relates to a fusion of at least one desired protein or polypeptide and at least one starch binding domain, as expressed in - or more generally as present in - in a plant or in any part of a plant, including the seeds, leaves, roots (including tuburous roots), tubers, stems, stalks, fruits, grains or flowers, and in particular the honey-producing parts of flowers, of a plant.

A further aspect of the invention relates to a genetic construct suitable for transforming a plant, comprising at least one nucleotide sequence encoding a desired protein or polypeptide combined with at least one nucleotide sequence encoding a starch binding domain.

Yet other aspects of the invention comprise a plant that has been transformed with a genetic construct as mentioned above, or a descendant thereof, and/or a plant that expresses a fusion as described above, preferably in conjunction with any starch (granules) present in or produced by said plant.

Further aspects of the invention will become clear from the description hereinbelow.

The protein or polypeptide that is expressed via the method of the invention can be any polypeptide or protein known per se, and is preferably an enzyme. The protein or polypeptide may or may not by itself have natural affinity for starch or starch granules. If the protein or polypeptide essentially does not have any affinity for starch (granules), or has only a very low affinity, the method of invention may be used to provide the protein or polypeptide with such affinity. If the protein or polypeptide has some natural affinity for starch (granules), such as enzymes that naturally interact with starch or starch granules, the method of invention may be used to increase said affinity, and/or to alter said affinity. For instance, it is known that starch may occur in different forms or states, which may for instance differ in crystallinity (i.e. amorphous vs crystalline) and/or in one or more other properties such as glucan content or degree of branching, and such different forms of starch may also form different parts or regions of one and the same starch granule. The method of the invention may therefore also be used to alter (such as increase or decrease) the affinity of enzymes for one or more such specific forms of starch, both in absolute terms as well as in relative terms (i.e. compared to the affinity for other forms of starch). In this way, the affinity of the enzyme for different starch granules and/or for different parts or regions of a single starch granule may also be altered (again both in absolute or in relative terms).

Therefore, a further aspect of the invention relates to a method for providing a protein or polypeptide with affinity for starch (granules), and/or for increasing the affinity of a protein or polypeptide for starch (granules), and/or for altering the affinity of a protein or polypeptide for starch (granules) comprising expressing the protein or polypeptide in a plant as a fusion with at least one starch binding domain.

More specifically, this aspect of the invention comprises the steps of:

a) combining a nucleotide sequence encoding the protein or polypeptide with at least one nucleotide sequence encoding a starch binding domain, so as to provide a

genetic construct encoding a fusion of the protein or polypeptide and the at least one starch binding domain;

b) transforming a plant with said genetic construct;

c) expressing said genetic construct in the plant.

The (gene(s) encoding) the desired protein or polypeptide may be derived from any source, including from plants, animals, fungi, algae, yeasts, bacteria and/or other micro-organisms, and may be homologous or heterologous to the plant in which the fusion is expressed.

Alternatively, (genes encoding) variants or mutants of such proteins or polypeptides may be used, such as those known per se in the art and/or obtainable via genetic manipulation techniques. These include mutant enzymes with altered properties compared to the enzyme from which they have been derived, such as altered substrate binding activity, altered substrate specificity, altered conversion properties and/or altered kinetic characteristics.

Furthermore, instead of the full protein or polypeptide as mentioned above, one or more fragments, parts, regions or domains thereof may be incorporated in and/or expressed as part of the fusions of the invention. Preferably, these fragments, parts, regions or domains are such that after expression, the fusion of the invention still provides the desired biological activity of the protein or polypeptide.

According to one preferred embodiment of the invention, the protein or polypeptide is an enzyme that can 'interact with' starch or starch granules, by which is meant that the enzyme can convert, modify, alter, degrade or otherwise influence the starch, the starch granule or the structure – and in particular the fine structure – and/or interactions thereof, and/or the physical and/or chemical properties of the starch (granule).

Some non-limiting examples of such ("starch altering") enzymes include, but are not limited to, different kinds of amylases (alpha-, beta, gluco-, iso-, etc.), CGTases, (neo)pullulanase, amylomaltase, glucan- and levansucrase, branching enzyme (such as glgB), fosforylating enzymes such as certain kinases, oxidative enzymes (such as oxidases and dehydrogenases), other starch-decorating enzymes (including those suitable for the transfer of sulfate and acetyl groups) and glycosyltransferases.

These enzymes may be obtained from any suitable source including bacteria, yeasts, fungi, algae and other micro-organisms including but not limited to *Escherichia*

coli, *Bacillus* (*subtilis*, *cereus*, *polymyxa*, *stearothermophilus*, *licheniformis*, *firmus/lentus*, *circulans*, *macerans*), *Aspergillus* (*niger*, *oryzae*, *kawachi*), *Klebsiella aerogenes*, *Streptomyces limosus*, *Pseudomonas* (*saccharophila*, *amyloderamosa*, *stutzeri*), *Clostridium* (*thermohydrosulfuricum*, *thermosulfurogenes*), *Microbacterium*, *Thermoanaero-bacterium thermosulfurigenes*, *Saccharomyces cerevisiae*, *Rhizopus oryzae*, *Streptococcus mutans*, *Leuconostoc mesenteroides*, *Neisseria polysaccharea and/or Aureobasidium pullulans*.

The enzymes may also be derived from higher forms of life including plants and animals. Some examples of plant-derived enzymes include starch synthases, starch-branching and -debranching enzymes (including isoforms thereof, such as BE-I and BE-II), disproportionating enzymes, putative potato kinase (sometimes also referred to as R1) etc., as well as the further enzymes mentioned in A.M. Smith et al. and C.Martin and A.M. Smith, above.

Other suitable starch-altering enzymes are for instance described in US-A-5,665,585.

In particular, the expression in a plant of a fusion of at least one starch binding domain and an enzyme that can interact with starch or starch granules as defined hereinabove will lead to (a transformed plant that produces) a starch (granule) of which at least one property has been altered, compared to the starch (granule) natively produced by said plant (in which the presence of the fusion as such is not taken into account).

More in particular, such expression of a "starch altering fusion" will lead to (a transformed plant that produces) a starch (granule) of which at least one of the properties 1-15 listed hereinbelow has been altered, compared to the starch (granule) natively produced by said plant (in which these properties and any alterations therein compared to the "wild type" starch can be determined in any manner known per se, including but not limited to the methods indicated below):

1. Morphology (microscopy [Kuipers et al. (1994) Plant Cell 6, 43-52; Edwards et al. (1999) Plant J. 17, 251-261])
2. Granule size distribution (Coulter multisizer [analysis follows the instructions of the manufacturer])
3. Amylose:amylopectin ratio (many different methods including iodine-staining combined with optical density measurements [Hovenkamp-Hermelink et al. (1988)

Potato Res. 31, 241-246], size-exclusion CL2B chromatography [Denyer et al. (1995) Plant Cell Environment 18, 1019-1026])

4. Molecular weight of the constituent polymers (size-exclusion CL2B chromatography [Denyer et al. (1995) Plant Cell Environment 18, 1019-1026])
5. Degree of branching (among others: debranching of gelatinized starch followed determination the chain length distribution of the forth-coming digest by high-performance size-exclusion chromatography [Kossmann et al. (1999) Planta 208, 503-511], high-performance anion-exchange chromatography [Safford et al. (1998) Carbohydrate Polymers 35, 155-168; Kossmann et al. (1999) Planta 208, 503-511], or MALDI-TOF mass spectrometry)
6. Different distribution of glycosidic linkages (enzymic digestion of starch with isoamylase, alpha- or beta-amylase [Colonna and Mercier (1984) Carbohydrate Research 126, 233-247; Safford et al. (1998) Carbohydrate Polymers 35, 155-168; Kossmann et al. (1999) Planta 208, 503-511], followed by chromatography to purify the obtained fragments [Safford et al. (1998) Carbohydrate Polymers 35, 155-168], and subsequent analysis by mass spectrometry and or NMR [Fontaine et al. (1993) Journal Biological Chemistry 268, 16223-16230])
7. Different decoration patterns with substituents, such as phosphate groups (several methods, including NMR [Blennow et al. (1998) Carbohydrate Research 307, 45-54; Safford et al. (1998) Carbohydrate Polymers 35, 155-168] and glucose-6-phosphate/total phosphate determination [Visser et al. (1997) Starch 49, 443-448])
8. Crystallinity (X-ray diffraction [Buleon et al. (1998) Macromolecules 31, 6605-6610; Bogracheva et al. (1999) Carbohydrate Polymers 39, 303-314])
9. Degree of cross-linking (X-ray diffraction [Buleon et al. (1998) Macromolecules 31, 6605-6610; Bogracheva et al. (1999) Carbohydrate Polymers 39, 303-314], differential scanning calorimetry [Visser et al. (1997) Starch 49, 443-448])
10. Gelatinization (differential scanning calorimetry [Visser et al. (1997) Starch 49, 443-448], Bohlin viscosimetry [Visser et al. (1997) Starch 49, 443-448])
11. Retrogradation (Bohlin viscosimetry [Visser et al. (1997) Starch 49, 443-448])
12. Solution properties such as viscosifying potential (Bohlin viscosimetry [Visser et al. (1997) Starch 49, 443-448])
13. Water-binding or swelling potential of the granules [Visser et al. (1997) Starch 49, 443-448]

14. Gel strength, adhesiveness, cohesiveness, elasticity and hardness (texturometer [Visser et al. (1997) Starch 49, 443-448]); and/or

15. Film-forming properties.

Preferably, the above properties, when mentioned via the method indicated, will be altered by at least 1%, preferably at least 5%, more preferably at least 10%, compared to the corresponding "wild type" starch (granule).

Some non-limiting examples of other proteins or polypeptides (i.e. non-enzymatic) that may be expressed in association with starch (granules) according to the invention include proteins or polypeptides derived from micro-organisms, plants or animals, such as receptors (such as estrogen receptors and in particular plant hormone receptors) and other structural proteins, such as protein "zippers".

According to one embodiment of the invention, the desired protein or polypeptide is not β-galactosidase or another reporter enzyme.

The term 'starch binding domain' or 'SBD' is well-known in the art, for instance from the references cited hereinabove, and is generally used to denote any part, region or domain of a protein or polypeptide, and in particular of an enzyme, that has natural affinity to (i.e. that binds to, attaches to, complexes with or otherwise associates with) starch or starch granules, or more generally with polymers of glucans.

Any naturally occuring starch binding domain, or any part or fragment thereof that still has affinity for starch (granules), may be used, as well as variants or mutants thereof.

As such, the starch binding domain(s) used in the invention may be derived from any protein or polypeptide known per se that contains one or more starch binding domains, including proteins or polypeptides derived from plants, animals, fungi, algae, yeasts, bacteria and/or other micro-organisms. The starch binding domains may be homologous or heterologous to the plant in which the fusion is expressed. Preferably, a starch binding domain of an enzyme is used, more preferably an enzyme derived from a bacterium, yeast, fungus or (other) micro-organism, or of a plant, such as GBSSI, which occurs in many different plants.

Some non-limiting examples of enzymes from which the starch binding domains may be derived are mentioned in the prior art indicated above, and may further include enzymes from bacteria, yeasts, fungi, or other micro-organisms such as the cyclodextrin glycosyl transferases ("CGTases"), for instance from *Bacillus circulans*,

Aspergillus awamori, *Aspergillus kawachi*, *Klebsiella pneumoniae* or *Bacillus staerothermophilus*; SBDs derived from thermostable enzymes, such as the CGTase of *Thermoanaerobacterium thermosulfurigenes;* glucoamylases, for instance from *Aspergillus niger* (which is reinforced by a disulfide bridge); glucoamylase from *Rhizopus oryzae*, alpha-amylase from *Streptomyces limosus*, beta-amylase from *Clostridium thermosulfurogenes*, maltogenic alpha-amylase from *Bacillus stearothermophilus*, maltotetraose-forming amylase from *Pseudomonas stutzeri*; as well as enzymes from plants or animals such as granule-bound starch synthase I (GBSSI), partially granule-bound SSI, SSII and SSIII, a putative kinase (R1), and other granule-associated enzymes, as well as engineered forms of such domains.

Other suitable starch binding domains include for instance those derived from the enzymes mentioned in US-A-5,665,585, as well as the natural starch binding domains and variants thereof described by Penninga et al., Lawson et al., Sorimachi et al., Svensson et al., Goto et al., Williamson et al., Chen et al. and Dalmia et al, above.

Although generally, all starch binding domains known per se can be used in the invention, including but not limited to those indicated above, it should be understood that for some applications of the invention, some starch binding domains may be preferred compared to others.

For instance, for the modification of the properties of (a) starch (granule), it may be that some types of starch binding domain (e.g. SBD) may direct the starch altering enzymatical activity fused with said starch binding domain towards the surface of the starch granule - for instance so as to alter one or more of the properties of and/or associated with said surface - whereas other types of starch binding domains may cause the starch altering enzymatical activity fused with said starch binding domain to be incorporated/enclosed within the starch granule.

In this way, the invention may not only make it possible to use a starch altering fusion as described herein to alter the properties of the starch (granule), but also to determine to at least some extend where said starch altering fusion effects its starch altering activity, e.g. within the starch granule and/or at the surface of the starch granule. In this respect, for a desired alteration of a property of the starch (granule), the skilled person will be able to select, based upon the teaching provided herein, both a suitable starch altering enzymatical activity as well as a suitable starch binding domain,

which allows said enzymatical activity to be effected as efficiently as possible and/or at the desired site (e.g. the surface of the starch and/or within the starch granule).

Also, it is to be understood that although it is preferred in the invention to use only those parts or regions of the abovementioned enzymes, proteins or polypeptides that form the "starch binding domain(s)", it is not excluded that one or more other parts or sequences of the original enzyme may also remain or be present in the fusions of the invention (i.e. attached to the starch binding domain). It may even be possible to use the full amino acid sequence of the original enzyme as a starch binding domain in the invention, i.e. as a fusion with the desired enzyme, protein or polypeptide. When such further parts or sequences are (still) present, these most preferably (no longer) show any biological activity per se. When a full sequence is used as a starch binding domain, this sequence has preferably been made devoid of biological activity (e.g. made catalytically inactive), although again the invention in its widest sense is not limited thereto.

Some preferred examples of enzymes that can be used as starch binding domains in the invention (i.e after they have been made catalytically inactive) include catalytically inactive GBSS I or gbSSII.

Generally, such starch binding domains are polypeptides of about 95 to about 105 amino acids amino acids, although their size is not essential in the invention. For instance, when catalytically active enzymes such as GBSSI or gbSSII or parts thereof are used, they may have a size of about 525-770 amino acids. For some specific applications, such large starch binding groups may be preferred, but generally smaller size SBD's (i.e. up to about 150 a.a.) will be preferred.

According to one embodiment, the starch binding domain used in the invention contains at least one of the minimal sequences shown in Figure 1, or a variant or mutant thereof, for instance in which one of the tyrosine residues in binding site 2 have been replaced by tryptophan.

Other suitable starch binding domains can be identified by means of sequence alignment of the above minimal sequences of Figure 1 with a known database, for instance an alignment program known per se such as BLAST or PC gene. In general, any domain or region of an enzyme that contains a sequence that has a sequence homology of more than 50%, preferably more than 70%, more preferably more than

90% with the abovementioned minimal sequence as can be used (in which a deletion or insertion is counted as a single mutation).

Preferably, any binding domain used in the invention has an affinity for starch (granules) (expressed as K_{ad}-value) of more than 10, preferably more than 15 ml/g, as measured by means of adsorption isotherm methodology as described by Chen et al, for instance in the Protein Engineering-, Biotechnol. Prog.- and/or Gene-references mentioned above. In general, this involves measuring the absorption of the binding domain to native starch at different concentrations of the protein (i.e. of the binding domain). After mixing the protein (for instance in concentrations ranging from 0,1 to 1,0 mg/ml) and the starch (for instance 0,1g) in a suitable aqueous medium (for instance 1 ml total volume), the mixture is shaken for a suitable period of time (for instance 20 min to 1 hr) at a suitable temperature (for instance 4°C), after which the mixture is subjected to centrifugation (for instance 17400 *g* for 20 min), after which the protein concentration of the supernatant is assayed and the amount of adsorbed protein is determined by subtraction. Values for the absorption constant (K_{ad}) can then be derived from the slopes of the linear adsorption isotherms, optionally by comparison with a reference protein.

Also, preferably, the entire fusion of the invention also has an affinity for starch (again expressed in terms of K_{ad} - value) of more than 10, preferably more than 15 mL/g.

With respect to the affinity for starch and the K_{ad}-value, it will further be clear to the skilled person that these may also be influenced by factors usch as the type of starch used, the presence of glycosylation, the size of the fusion, the type of linker (if any) etc.. Also, other assays for determining the affinity of a protein to starch have been described in the art, and these can also be used to identify suitable domains or regions.

In another embodiment of the invention, and as an alternative for starch binding domains, domains or regions with affinity for other glucan polymers such as cellulose, amylose, or amylopectin, glycogen, mutan, dextran, nigeran, pullulan, or affinity for fructan, pectin, xylan, or (mixed linkage) betaglucan polymers may be used. These include for instance enzymes or proteins for glucan polymers other than starch such as the glucan binding domains of sucrases, cellulose binding domains (which however have no or very low affinity for starch, and are therefore less preferred), granule-bound starch synthase I or part thereof, as well as engineered sucrose porin, maltose binding

protein, maltoporin and/or lamB from *E.coli*, which have affinity for maltose/maltodextrin (The latter, however, have the disadvantage of a large size, i.e. about 400-600 amino acids, and also have little or no affinity for so-called raw starch).

Such cellulose binding domains, maltose binding proteins etc. can be used/expressed in a fusion of the invention analogously as described herein for the starch binding domains, to provide fusions with affinity for, and/or that can associate with, cellulose and maltose/maltodextrin, respectively. However, the use of starch binding domains, so that the resulting fusion can associate with starch (granules), is much preferred.

In the fusions expressed according to the invention, the desired protein or polypeptide may be fused directly with the one or more starch binding domains, or via a linker sequence, i.e. a sequence of 1-100, preferably 4-60 (optionally glycosylated) amino acids that connects the protein or polypeptide with the one or more starch binding domains. The linker sequence may also act as a "hinge" and/or spacer, for instance in order to ensure that the presence of the starch binding domain or the binding thereof to the starch (granule) does not interfere with or detract from the desired activity or properties of the enzyme, protein or polypeptide (and visa versa).

In principle, any natural or synthetic amino acid sequence can be used as a linker, preferably a sequence that essentially does not interfere with either the affinity of the starch binding domain for the starch (granules), or with the desired activity of the enzyme.

Some non-limiting examples of suitable linker sequences include:

- the 40 AA linker sequence used in Trichoderma, comprising a flexible part and glycosylated rigid part;
- naturally occurring or synthetic sequences of alternating Pro-Thr;
- linker sequences containing major amount glycine residues or of (optionally O-glycosylated) serine or threonine residues.

Other suitabe linkers are for instance described by P. Argos, J. Mol. Biol. (1990), 211, p. 943-958.

The protein or polypeptide may also be bound to the one or more starch binding domains via an amino acid sequence that can be cleaved, i.e. chemically or preferably enzymatically. Examples are amino acid sequences that provide enzymatic cleavage

site for enzymes such as thrombin, factor Xa, and collagenase can be mentioned. Such a sequence may also form part of a larger linker sequence as described above.

In the fusions of the invention, the one or more starch binding domains may be at the N'-terminus of the fusion, at the C' terminus of the fusion, or – if two or more starch binding domain are present - both. If a starch binding domain is used that is naturally (i.e. in the enzyme from which it has been derived) is at the C' terminus (or N' terminus), it is preferably also at the C' terminus (or N' terminus, respectively) of the fusion of the invention, although the invention is not limited thereto.

Also, when a fusion to the invention contains two or more starch binding domains, these may be the same or different (such as different domains from the same enzyme or domains from different enzymes/sources) and may -if present at the same terminus - also be fused directly or via a linker sequence, that again may also act as a hinge and/or spacer.

Furthermore, although usually not preferred, it is also possible that a fusion of the invention (a "bifunctional fusion") contains two or more of desired enzymes, proteins or polypeptides, which may be fused directly, via a linker sequence, or via a sequence encoding one or more starch binding domains (besides any further starch binding domains that may be present at the 3' and/or 5' end). For instance, there are cellulases and xylanases with "internal" cellulase binding domains, which may be included "internally" between two catalytic centers of the bifunctional fusion.

The fusions of the invention are obtained by expression in a plant *in vivo* of a nucleotide sequence (genetic construct) that codes for the fusion of the invention. For this purpose, the plant may be or may have been transformed with said genetic construct, or may be a descendant (such as obtained via sexual or asexual multiplication, including crossing and/or other breeding techniques) of a plant that has been transformed with such a genetic construct, and that has inherited the genetic construct.

The plant may be any monocotylous or dicotylous plant in which the fusions can be expressed, but is preferably a plant that naturally contains or produces starch, and more preferably a plant that contains or produces starch granules, either throughout the entire plant or in any part thereof, including seeds, leaves, roots (including tuburous roots), tubers, stems, stalks, fruits, grains or flowers, and in particular the honey-producing parts of flowers; and such a plant is referred to herein as a 'starch granule

producing plant'. As mentioned above, said starch granules will usually be associated with or present in specific organelles of the plant cell, and in particular the plastids, such as chloroplasts, amyloplasts and/or chromoplasts.

Some preferred non-limiting examples of starch granule producing plants suitable for use in the invention include economically important crops such as potato, sweet potato, cassava, pea, taro, sago, yam, and/or cereals such as rice, maize, wheat and barley; of which potato, sweet potato, maize and wheat are especially preferred.

The starch granule producing plant may also be a plant, and in particular a genetically modified plant, that as such already produces a modified starch, such as transformed potato plants producing mutant amylose free ("amf") starch. For some applications, the use of a plant that produces starch granules that contain pores may be useful, in particular when the further use of the starch granules associated with the fusions of the invention involved diffusion of compounds in and out of the granules. For instance, some cereal starches are known to have natural pores. Also, the use of small granules, again as for instance in cereal starches, may also be advantageous with respect to diffusion of substances into the starch granule. Alternatively, a (pre)treatment with an enzyme that make pores or holes in starch granules, such as those of microbial origin, may be used.

A genetic construct encoding a fusion of the invention may be obtained by 'combining' the nucleotide sequence(s) encoding the at least one desired protein or polypeptide with at least one nucleotide sequence that codes for a starch binding domain, optionally with or via one or more sequences that encode a linker sequence as described above, in such a way that expression of the combined sequences in the desired plant leads to the formation of the fusion.

Generally, this involves covalently binding the nucleotide sequences in the same reading frame and in the same orientation, and in the correct order from the 5' end to the 3' end. This can be carried out using genetic manipulation techniques known per se, such as those described in Sambrook et al, "Molecular Cloning: A Laboratory Manual" (2nd.ed.), Vols. 1-3, Cold Spring Harbor Laboratory (1989); or F. Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York (1987)

The one or more sequence(s) encoding the starch binding domains can be provided synthetically using known DNA synthesis techniques, but are preferably

isolated from the organism from which the starch binding domain has been derived (i.e. in which it naturally occurs). Similarly, the sequence encoding the protein or polypeptide is also preferably isolated from a suitable biological source, and as such may be a nucleotide sequence encoding the mature protein, or a nucleotide sequence encoding a precursor thereof, that can be converted into the mature protein or polypeptide by post-translational modification(s) in the plant (i.e. as part of the encoded fusion).

The genetic construct encoding the fusions of the invention may further contain all other elements known per se for nucleic acid sequences or genetic constructs, such as promoters or other control elements, terminators, translation or transcription enhancers, integration factors, signal sequences, selection markers, etc., that are preferably suited for use in (the transformation of) the host plant. The sequences that encode these further elements of the construct may again be either isolated from a suitable biological source, or provided synthetically. Examples of suitable elements are for instance described in DE-A-195 34 759, WO 91/19808, US-A-5,349,123, US-A-5,750,875 and WO 92/14827.

The one or more nucleotide sequences encoding the further elements of the construct can again be combined with the nucleotide sequence encoding the fusion in a manner known per se, such as described in Sambrook et al., Ausubel et al., DE-A-195 34 759, WO 91/19808, US-A-5,349,123 or US-A-5,750,875.

Preferably, they are combined in such a way that – after transformation - the construct can be used for the expression of the fusion in the desired plant. Generally, this involves combining the control elements and any further elements with the sequence encoding the fusion in an operable manner, i.e. in the same reading frame and in the same orientation, and in the correct order from the 5' end to the 3' end.

The promoter can be any promoter that is able to control/induce the expression of the fusion in the intended plant, including constitutive and inducable promoters, and may be homologous or heterologous to said plant.

Also, a promoter may be used that directs the expression of the fusion to a specific part or tissue of the plant, and in particular to a tissue or part of the plant where starch (granules) are formed or present, including the including seeds, leaves, roots (including tuburous roots), tubers, stems, stalks, fruits, grains or flowers. and in particular the honey-producing parts of flowers, etc.. Furthermore, a promotor may be

used that induces expression of the fusion during a specific period in the life cycle of the plant. For instance, in potato, a promotor may be used that specifically directs the expression of the fusion in or to the tuber, and/or that allows for expression only during the time the plant forms its tubers.

Examples of suitable promoters include the CaMV promotor, GBSS promotor, patatin promotor, Ubiquitin promotor, ST1 promotor, TR1 promotor, napin promotor, as well as for instance the promoters described in DE-A-195 34 759, WO 91/19808, US-A-5,349,123, US-A-5,750,875 and WO 92/14827. For specific expression of foreign genes in potato tubers, reference is made to for instance EP 0 375 092 and Rocha-Sosa et al., EMBO J. 8, 23-29 (1989).

The construct of the invention may also comprise one or more sequences that encode signal proteins, including pre-, pro- of prepro-sequences. These usually precede the sequence encoding the fusion, such that the fusion is expressed as a (further) fusion with these signal proteins. The signal sequence may ensure any post-translational modifications required for the formation of the mature fusion (i.e. of the protein/polypeptide and/or the starch binding domain(s) part thereof), and/or may specifically direct the expressed fusion to a desired part or organel within the plant or plant cell, and in particular to the starch granule(s). In particular, signal sequences for plastide targeting, such as for amyloplast, chloroplast or chromoplast targeting can be used, or signal sequences for targeting the vacuole. Some non-limiting examples thereof include the small subunit RuBisCo, GBSS transit peptides and sporamine transit peptide.

According to one preferred embodiment, the genetic construct encoding the fusion is preferably in a form suitable for transformation of a plant, such as a vector or plasmid. As such, the construct is preferably such that upon transformation it is incorporated into the (genomic) DNA of the plant. However, the construct may also be in any other form that can provide for expression of the fusion in the plant, and that preferably also can be stably and/or independantly maintained and/or replicated in the plant, and/or inherited from one generation of the plant to the next. The construct is preferably further in a form that can be stably and/or independantly maintained and/or replicated in any organism to be used for constructing or selecting the construct and/or to be used in transforming the plant, such as *Agrobacterium*.

A further aspect of the invention therefore relates to a bacterium, virus or other organism suitable for transforming a plant, containing a genetic construct as defined above, and preferably capable of transferring said construct into a plant. The organism may for instance be a strain of *Agrobacterium*.

After construction, the construct is transformed into the desired plant, preferably a starch granule producing plant as defined above. Any technique for the transformation of a plant known per se can be used. Examples thereof include transformation using *A. tumefaciens* or *A. rhizogenes*, electroporation of tissues and/or protoplasts, particle bombardment, use of virusses for DNA delivery, etc., as well as the techniques described in DE-A-195 34 759, WO 91/19808, US-A-5,349,123, US-A-5,750,875 and WO 92/14827

After transformation, a plant is (re)generated from the transformed cells or tissue and the construct is expressed in the plant or part thereof, optionally upon induction thereof in a suitable manner.

The invention therefore also relates to a method for providing a plant that expresses a fusion as described above, comprising at least one step of:

a) transforming a plant with a genetic construct as described above, such that said genetic construct is expressed in the plant or at least part thereof;

and optionally further comprising at least one step of:

b) providing descendants and/or further generations of the thus transformed plant, for instance via sexual or asexual multiplication, including crossing and/or other breeding techniques.

The invention also relates to seeds, tubers, seedlings, stakes (e.g. for cassava) or other cultivating material of such a transformed plant.

Upon expression, the fusion will usually become 'associated with' any starch granules present in the plant, by which is meant that the fusion attaches to, binds to, complexes with or otherwise combines with the starch granule, i.e. via the one or more starch binding domains present in the fusion. Said association may be such that the fusion is present on the surface of the starch granule, and/or incorporated into (such as by encapsulation or enclosure) the starch granule (e.g. during the biosynthesis thereof). The association(s) thus obtained will be collectively referred to hereinbelow as the 'complex'. One preferred embodiment of the invention concerns these complexes,

methods for their preparation by expression in a plant, and plants that express these complexes.

As mentioned above, the starch granules in a plant will usually be present in or associated with specific organelles within the plant cell, and in particular the plastids, such as the amyloplasts, chloroplasts or chromoplasts. It should be understood that when in the present description and claims mentioned is made of a "starch granule", this is also meant to include (the starch granules as present in) these organelles. The term "complex" as mentioned herein therefore also includes complexes of fusions of the invention and such organelles.

Therefore, in another aspect, the invention relates to a method for producing a complex of at least one protein or polypeptide and a starch granule, comprising at least one step of:

a) expressing the protein or polypeptide as a fusion with at least one starch binding domain, in a plant that contains or forms starch granules;

and optionally comprising at least one further step of:

b) isolating the protein or polypeptide from the plant or any part thereof as a complex of the fusion and the starch granule.

A further aspect of the invention relates to a complex, comprising a fusion of a protein or polypeptide and at least one starch binding domain, associated with a starch granule.

In particular, this aspect of the invention relates to such a complex as expressed in/obtained from a plant via the method described above.

Yet another aspect of the invention therefore relates to a method for providing a plant that can produce a complex of a fusion as described above and a starch granule, comprising at least one step of:

a) transforming a starch granule producing plant with a genetic construct as described above, such that said genetic construct is expressed in the plant or at least part thereof;

and optionally further comprising at least one step of:

b) providing descendants and/or further generations of the thus transformed plant, for instance via sexual or asexual multiplication, including crossing and/or other breeding techniques.

The invention further relates to the complex-producing transformed plants thus obtained, or any descendant thereof, as well as cultivation material of said plant, including seed, tubers, stakes or seedlings.

Usually, and preferably, the fusion and the starch granule will already associate *in vivo*, so that they can be obtained/isolated together, using techniques known per se for the isolation of the starch granules from the plant or plant material. However, the invention is not limited thereto. For instance, the complex may also be formed during or as a result of the isolation/further processing of the plant or plant material, for instance when the fusion is expressed in a part of the plant (cell) separate(d) from the starch granule. In yet another embodiment, starch granules, optionally combined with a further carrier or matrix, can be used to selectively isolate the fusion from the plant (material), i.e. by a method comparable to an affinity technique.

Again, instead of isolating the starch granules, it may be easier and/or advantageous to isolate the organelles in which the starch granules are present, again in a manner known per se, so as to provide complexes of the starch granule containing organel and the fusion of the invention. These may also be used as such in any subsequent application(s).

The above aspect of the invention is particularly suited for producing a desired protein or polypeptide in for instance potato tuber, cassave root, sweet potato tuber, grains of maize, wheat and barley, as well as in peas, etc.. After the tubers, roots or grains produced by the plants expressing the fusions have been harvested, the protein or polypeptide can be conveniently isolated therefrom as a complex with the starch granules. This aspect thus provides for a very efficient production and isolation of any desired protein or polypeptide, and may be used to produce such a polypeptide or protein in major and/or commercial amounts.

The complex thus obtained may be processed further, for instance for further purification, in which the fact that (essentially only) the desired protein or polypeptide is present as a complex with the starch granules may be used with advantage. The fusion may also be separated from the starch granules, and/or the fusion may be cleaved (i.e. at a suitably situated enzymatic cleavage site as described above) in order to provide the desired protein or polypeptide.

Compared to the expression of fusions in bacterial expression hosts such the *E.coli* strains described in the art, expression of the desired proteins or polypeptides

according to the invention in (edible) plant material such as potato tubers may be advantageous from a safety standpoint. Also, expression of the desired protein or polypeptide in a plant host compared to a bacterial host may be advantageous for some applications, for instance when a bacterial host does not carry out all desired post-translational modifications or conversly degrades or otherwise detracts from the desired protein or polypeptide. For such applications, the method of the invention may provide a valuable alternative.

According to another preferred embodiment of the invention, the plant used to express the fusions of the invention is a starch producing plant, in particular a starch granule producing plant, and the protein or polypeptide expressed as part of said fusion according to the invention is an enzyme that can interact with starch or starch granules.

In this embodiment, after expression, the fusions preferably associate (either *in vivo* and/or during processing of the plant or plant material) with the starch (granules) via the one or more starch binding domains, after which the enzyme can interact with the starch (granule), for instance to convert, modify, alter, degrade or otherwise influence the starch, the starch granule or the (primary) structure or interactions thereof, resulting in (a plant or plant material that can be used to provide) a modified starch, i.e. a starch different from the starch naturally provided by the plant in at least one property thereof, and in particular in one or more of the properties "1-15" mentioned above.

In particular, in this aspect of the invention, the enzyme that can interact with the starch (granules) is an enzyme that does not occur naturally in (i.e. that is heterologous to) the original starch producing plant, but is for instance an enzyme derived from another plant or from a bacterium, fungus or (other) micro-organism as described above.

In this way, the invention can be used to provide a transformed plant that *in vivo* produces starches that are modified or altered (i.e. compared by the starch naturally produced by the original starch producing plant).

Therefore, yet another aspect of the invention relates to a method for providing a plant that contains or produces a modified starch and/or modified starch granules, comprising at least one step of:

a) transforming a starch producing plant, in particular a starch granules producing plant, with a genetic construct comprising at least one nucleotide sequence encoding an enzyme that can interact with starch and/or starch granules and at least

one nucleotide sequence encoding a starch binding domain, such that said genetic construct is expressed in the plant or at least part thereof;

and optionally further comprising at least one step of:

b) providing descendants and/or further generations of the thus transformed plant, for instance via sexual or asexual multiplication, including crossing and/or other breeding techniques.

The invention further relates to the transformed plant producing modified starch (granules) thus obtained, or any descendant thereof, as well as cultivation material of said plant, including seed, tubers, stakes or seedlings.

This aspect of the invention also comprises a method for producing a modified starch and/or modified starch granules, comprising at least one step of:

a) cultivating a transformed plant that produces a modified starch and/or modified starch granules as described above, or a descendant thereof:

and optionally further comprises at least one step of:

b) isolating the modified starch or starch granules from the transformed plant or from any part thereof, such as its seeds, leaves, roots (including tuburous roots), tubers, stems, stalks, fruits, grains or flowers.

This aspect of the invention can in particular be used to provide potato, sweet potato, cassava and/or cereals such as maize, rice, wheat, and barley or other economically important crops that produce modified starches, i.e. in their tubers or seed, and such tubers or seeds containing modified starches form a further aspect of the invention. Another aspect of the invention resides in the modified starches thus obtained.

Alternatively, the invention may be used to provide starch granules that already contain one or more enzymes that can interact with starch, or plant material that contains such starch granules, i.e. attached to or incorporated within the starch granule via the one or more starch binding domains. After harvesting of the plant, plant material and/or starch granules, the enzymes may then be used *in vitro* to alter the properties of the starch (granule), to provide a modified starch.

For some applications, this embodiment may offer advantages over the *in planta* production of modified starches, for instance when specific conditions (such as temperature, pH optimum, the presence of certain co-factors, etc) are required for the starch converting activity of the enzyme, and/or when greater control over the

enzymatic starch conversion(s) is desired. For instance, enzymes such as thermostable α-amylases and isoamylase may have an optimal temperature range for conversion that may not be achieved *in planta* (i.e. in the field); a suitable temperature can then be applied during subsequent processing of the harvested plant material.

In a sense, this embodiment combines features of the two aspects of the invention described above, in that on the one hand the invention is used to express/provide a "complex" of a starch (granule) and a fusion of a starch-converting enzyme and one or more starch binding domains; whereas on the other hand the enzymatic activity present in the complex thus obtained is then used – through post-harvesting modification(s) - to provide a modified starch.

Furthermore, and analogous to the above described interaction with the starch (granules), the enzymatic activity expressed as part of the fusion may also convert, modify, alter, degrade or otherwise influence any other compound(s) present within the plant, or any other part(s) or biological function of the plant. The enzymatic activity may also provide for the *in vivo* synthesis of one or more desired compounds within the plant, which may or may not occur naturally in the original plant.

Therefore, in its broadest sense, this embodiment of the invention may be used to provide the plant with (i.e. to express within the plant) any desired enzymatic and/or biosynthetic activity, in association with any starch (granules) present within the plant.

According to yet another embodiment of the invention, only a nucleotide sequence encoding one or more starch binding domains as described above is expressed in a plant. According to this embodiment, the starch binding domain, which usually will be heterologous to the plant, upon expression preferably associates with any starch (granules) present in the plant, so as to make the starch granules less accessable to or a less favorable substrate or one or more starch converting enzymes that are naturally present in the plant. More generally, the starch binding domains thus expressed may compete with the native starch converting enzymes present in the plant (i.e. for "space" on the starch granules), thus influencing the native starch conversion processes.

In this way, the method of the invention can be used to inhibit, alter, modify or otherwise influence one or more of the native biosynthetic pathways in the plant involved in the starch (granule) biosynthesis or metabolism, which again can result in (a plant producing) a modified starch. Also, the interaction of the starch binding domain(s) and the starch (granule) can again lead to an alteration or modification of the

starch granule, such as changes in the (primary) structure, in the amylose content, in the crystallinity, etc..

Also, it should be noted that in this aspect of the invention, the nucleotide sequence may encode a single starch binding domain or two or more starch binding domains, which may be the same or different. Also, when such a nucleotide sequence encodes two or more (the same or different) starch binding domains, these domains may be fused or linked to each other, e.g. directly or via a suitable linker, including but not limited to those mentioned above for the fusions of the invention. The expression in a plant of such "linked" starch binding domains may for instance lead to starch (granules) which are crosslinked - e.g. to a larger extend compared to the native starch (granule) - by these linked starch binding domains, and/or may lead to one or more other alterations in the properties of the starch (granule), e.g. as mentioned above.

This aspect of the invention therefore generally comprises transforming a plant with a nucleotide sequence that codes for at least one starch binding domain, such that said starch binding domain is expressed in the plant, and preferably associated *in vivo* with any starch (granules) present in the plant or in any part thereof.

The nucleotide sequence is preferably in the form of a genetic construct as described above (but containing only the one or more sequences encoding a starch binding domain, optionally linked via a linker sequence, and any further elements known per se for such constructs as described above). Also, as more generally mentioned above, the 'starch binding domain' used in the may also include parts of the enzyme, protein or polypeptide from which the domain was derived, or may even be a full protein that has been made catalytically inactive, such as catalytically inactive GBSS I or the partially granule bound SSII (also sometimes referred to as gbSSII).

Therefore, yet another aspect of the invention relates to a method for providing a plant that contains or produces a modified starch and/or modified starch granules, comprising at least one step of:

a) transforming a starch producing plant, in particular a starch granules producing plant, with a genetic construct comprising at least one nucleotide sequence encoding a starch binding domain, such that said genetic construct is expressed in the plant or at least part thereof;

and optionally further comprising at least one step of:

b) providing descendants and/or further generations of the thus transformed plant, for instance via sexual or asexual multiplication, including crossing and/or other breeding techniques.

The invention further relates to the transformed plant producing modified starch (granules) thus obtained, or any descendant thereof, as well as cultivation material of said plant, including seed, tubers or seedlings.

This aspect of the invention also comprises a method for producing a modified starch and/or modified starch granules, comprising at least one step of:

a) cultivating a transformed plant that produces a modified starch and/or modified starch granules as described above, or a descendant thereof;

and optionally further comprises at least one step of:

b) isolating the modified starch or starch granules from the transformed plant or from any part thereof, such as its seeds, leaves, roots, tubers, fruits, etc..

Again, this aspect can in particular be used to provide provide potato, cassava, sweet potato, taro, sago, yam and/or cereals such as rice maize, wheat, barley or other economically important crops that produce modified starches, i.e. in their tubers or seed, and such tubers or seeds containing modified starches, from a further aspect of the invention. Another aspect of the invention resides in the modified starches thus obtained, optionally in the form of a complex with one or more starch binding domains as obtained through expression of the above genetic construct.

Some non-limiting, preferred practical applications of the invention include:

- Production of amylose-free starch, in particular amylose-free potato starch. Introduction of starch-binding domains, or any other protein binding to starch granules, may be used as an alternative for antisensing starch-converting enzymes, such as the GBSS I gene (or for mutating the GBSS I gene), to obtain amylose-free potato starch. In the *amf* mutant and the antisense GBSS I potato plants down-regulation of the amylose content occurs at the DNA and RNA level, respectively. In the SBD-expressing potato plants, reduction of the amylose content is based on competition between the expressed SBDs and GBSS I.

  The inhibition of the starch-converting enzymes may be further manipulated (i.e. increased) by using tandem SBDs (instead of a single SBD) separated by an appropriate linker peptide. Depending on the affinity for raw starch of the SBD in

comparison with GBSS I, a single SBD or a series of more than two SBDs may achieve an amylose-free potato starch.

- Modification of starch fine structure *in planta*.

Modification of the fine structure of starch can be achieved *in planta* by concentrating certain enzymes, equipped with an SBD, in the granule, or at the granule surface. Thus, next to targeting polypeptides to the amyloplast, also targeting within the amyloplast can be achieved. For instance, the use of *Escherichia coli* glgB or a potato kinase (sometimes referred to as R1) fused with an SBD can increase the degree of branching and phosphorylation of the starch to a larger extent than the non-engineered proteins.

Generating (new) transgenic starches, which either may have different functional properties compared to the existing ones (such as increased freeze-thaw stability or altered rheological properties), or which may be regarded as a better precursor for derivatization processes than the WT or amf potato starch.

- Starch modification *in vitro*.

In addition to modification *in planta*, (further) modification of the fine structure of the starch may take place during or after processing of the harvested plant (material). By introducing a protein in or on the surface of the starch granule, a complex of the invention that serves as a kind of 'precursor starch' can be obtained, which can be converted to the starch of interest after extraction of the starch from the plant (material). Thus, in this embodiment, the actual (or full) modification of the starch may not or may only in part be achieved *in planta*. The advantage of this is that (further) modification of the starch can take place under more controlled, and/or more extreme conditions than encountered in the plant *in vivo*, and/or in the presence of compounds such as reactants or co-factors which are not present in the amyloplast.

As an example thereof, the introduction of an oxidase/dehydrogenase as a fusion with one or more starch binding domains can be mentioned, yielding a more reactive starch after appropriate incubation *in vitro*. In this way, the derivatized starch can be obtained in a much more environmentally friendly manner than when chemical methods were applied.

- Starch granules with immobilized enzymes.

In this application, a foreign enzyme is introduced in or onto the starch granule, with the objective of using the granules as a carrier of enzymes. An enzyme of choice is fused to a SBD (with linker), and this fusion protein is incorporated into the starch granule during starch biosynthesis. Subsequently, these granules can be used to catalyze certain *in vitro* conversions in which reactants and products diffuse in and out of the granule. Such a procedure allows a simple separation of enzyme and products. In addition the enzyme can be re-used.

- Specialty support for affinity chromatography.
  In this application, a protein without a catalytic activity is introduced in or onto the starch granule during starch biosynthesis. The SBD can be fused to any kind of receptor, and the forth-coming starch can subsequently be used as a support for affinity chromatography, i.e. small molecules can be specifically absorbed from complex mixtures such as culture filtrates or plant extracts, and eluted after several wash steps.
- Molecular pharming of industrial enzymes.
  In this application the starch granule also serves as an affinity support, but in a different context. Industrially relevant enzymes are fused to a SBD (with linker), and can be produced in, for instance, the vacuole of plant cells. Upon disruption of the tissue, the enzymes are released from this compartment, and are able to contact the surface of granules released from the amyloplast. After washing, the fusion proteins may be eluted from the granules with a maltodextrin solution. It is also possible to release the enzyme (without SBD) with a highly specific protease.
- Production of cross-linked starches or starch granules.
  In this application, protein-carbohydrate interactions (SBD-starch) are combined with protein-protein interactions (for instance, a leucine 'zipper').
  Two possible uses thereof are for instance in reinforcing the granule and/or in creating a stronger starch gel after the granules are gelatinized.
  For reinforcing the granule, a leucine zipper domain may be flanked by one or two SBDs. The zipper domains can create a second network in the granule, which is connected to the first (starch) by the SBD(s). Possibly, the melting temperature of the granule can be changed in this way. Reinforcing the starch structure in this way may also be useful for providing complexes of the invention that can for example

be used in high temperature conversions. (Mutant starches like maize amylose-extender starch may also be useful in this respect)

For creating a stronger starch gel after gelatinization, a zipper domain may be flanked by a SBD on one side and a Maltose Binding Protein or 'MBP' on the other. The zipper domain is anchored in the granule by a SBD. After gelatinization, the zipper domains are connected, and this new network is attached to the starch network by MBPs. Gels with new properties may be realized in this way. In this aspect, the thermostability of the MBP may be of importance.

The invention will now be further illustrated by means of the Experimental Part given hereinbelow, as well as the Figures, in which:

- Figure 1 shows the amino acid sequence for some minimal sequences for starch binding domains suitable for use in the invention;
- Figures 2B-2H schematically show some examples of genetic constructs according to the invention containing reporter genes, with Figure 2A showing the vector $pBIN19^{PTT}$ used as the starting material for the construction of these genetic constructs..

Experimental Part

A. Introduction

Starch is an important storage material in many plants, such as potato, sweet potato, cassava, pea, yam, taro, sago and cereals such as maize, rice, and potato. It is deposited as crystalline granules which generally consist of two polysaccharides, amylose (generally 20-30%) and amylopectin (70-80%). Amylose is an essentially linear molecule which is composed of (1→4)-linked α-D-glucopyranosyl (α-D-Glcp) residues. Amylopectin is a highly branched molecule composed of a collection of α-(1→4)-glucan chains which are connected by α-(1→6)-linkages (the branch points). It is believed that branching occurs at regular intervals, in such a way that clusters of sidechains are formed. The sidechains can interact laterally with each other which is presumably the basis for the crystalline nature of the granule.

Current models explain granule growth by addition of single Glc residues to the non-reducing ends of a nascent amylopectin molecule, a reaction catalyzed by synthases. Once the glucan chains have reached a certain length, they are thought to be

rearranged with the help of a number of enzymes such as branching enzyme (BE), debranching enzyme (R-enzyme), and presumably also disproportionating enzyme (D-enzyme). As far as is currently known, starch-producing plant species can possess several isoforms of starch synthases (GBSS I, GBSS Ib, gbSS II, SSS I, and SSS III; GB=granule-bound, gb=partially granule-bound, S=soluble) and BE (BE I, BE IIa, and BE IIb).

The major differences between the potato starch synthase isoforms are the length of the N-terminal extension, and the location of expression in the plant. For SSS III the N-terminal extension is 780 amino acids long, for gbSS II 275. The C-terminal part of these two enzymes, SSS I, and GBSS I is very similar. Three regions in particular, termed box I to III, are highly conserved. Box I contains the "KTGGL" motif, which is the putative ADP-glucose binding site. The function of the other boxes is unknown. GBSS I and gbSS II are expressed in stolon and tuber, SSS I only in leaf, and SSS III in both tuber and leaf. The amino acid sequence of potato BE I and BE II is highly conserved, except at the extremities of the protein. BE II has a flexible N-terminal extension of approximately 120 amino acids which is absent in BE I. BE I has a C-terminal extension of 110 amino acids which BE II lacks. Contrary to maize, only two isoforms of BE have been found in potato sofar. It should be noted that BE I is much more abundant than BE II in potato tubers. In maize three isoforms have been reported (BE I, IIa, and IIb). The isoforms BE IIa and BE IIb of maize are very similar with respect to their amino acid sequence (except for the c. 50 N-terminal amino acids). However, these isoforms seem to be expressed in different tissues.

Although the primary structure of many synthases and BEs has been documented, this is not the case for the biochemical properties of these enzymes. Based on observations in certain mutant backgrounds, there is some evidence suggesting that each synthase plays a particular role in elongating sidechains of a specific length. Further, GBSS I is the only synthase involved in the synthesis of amylose. Next to (slightly) different catalytic properties, it is expected that the ability of synthases to bind to starch granules is determinative for their role in the biosynthesis process. GBSS I is found predominantly in the starch granule, whereas SSS III is found predominantly in the soluble phase. The gbSS II and SSS I seem to hold intermediate positions. The isoforms of BE seem to display a somewhat different mode of action. BE II prefers to transfer shorter glucan chains to an acceptor substrate than BE I. In addition, other

properties of the BEs might determine their specific roles in the biosynthesis process. For instance, BE II seems to associate more strongly with starch granules than BE I. In fact, one might say that BE II is partially granule-bound (maybe gbBE would be a more appropriate name). It is unknown whether its starch-binding ability is mediated through the N-terminal extension (which is typical for BE II).

Over the years, the level of several of the synthase or BE isoforms has been down-regulated by mutation or antisense technology. This large amount of data suggests that decreasing the level of granule-bound enzymes has more severe consequences for the starch granule architecture than that of their soluble isoforms. The presence of GBSS I determines whether or not amylose is deposited in starch granules. Knocking out SSS III expression in potato leads to a different granule morphology (T-shaped cracks and clusters of small granules). However, starch content of the tubers, the granule size, and the ratio of amylopectin and amylose remain more or less unaltered. Inhibition of gbSS II expression in potato does not result in large changes in starch content, morphology or composition. However, mutation of the pea gbSS II gene can result in an altered granule morphology and amylopectin structure.

The impact of downregulating a particular synthase may be related to how much this isoform contributes to the total amount of synthase activity in the crop. However, this may be difficult to assess. BE I is the most abundant BE isoform in potato tuber, and it resides predominantly in the soluble phase. Antisensing the gene encoding this enzyme effectively removes the BE activity from the tuber juice, but surprisingly, the starch structure is not affected by this. However, decreasing the level of BE II protein (which is at least partially granule-bound) in potato tuber by antisense technology dramatically reduces the number of α-(1$\rightarrow$6) branchpoints in the starch (and probably also the starch content of the tuber), and consequently the functional properties of the starch. The starch produced in this way shares some characteristics with the amylose-extender mutant in maize, in which the gene encoding BE IIb is inactivated.

According to the invention, soluble enzymes could be made granule-bound; and techniques can be provioded make other (non-)biosynthetic enzymes granule-bound.

Protein-carbohydrate interactions are not understood with respect to starch biosynthetic enzymes (or granule-boundness), but the ability to bind to crystalline particles is a common feature of many cellulose- or starch-degrading enzymes. In general, these enzymes are composed of two or more domains which are connected by

linker peptides. One of these domains contains the catalytic function of the protein, whereas other domains are involved in anchoring the proteins to the water-insoluble polysaccharide matrix. The so-called binding-domains (BDs) are usually relatively small (30 to approximately 160 amino acids) compared to the catalytic domains (>200 amino acids). As a result of this, the 3D structures of a large number of BDs have been solved. These studies have provided a rather detailed picture of how a protein interacts with carbohydrates. Further, a number of candidate amino acid residues which are presumably involved in binding the polysaccharide could be indicated. In some cases the importance of these residues for binding was verified by site-directed mutagenesis experiments. The interaction of cellulose-binding domains (CBDs) with cellulose and of (raw) starch-binding domains (SBDs) with starch granules will be further discussed hereinbelow.

Unlike CBDs, the amino acid sequences of SBDs seem very well conserved among different enzymes (α-amylase, ß-amylase, glucoamylase, cyclodextrin glycosyltransferase [CGTase], etc.), as well as among different species (*Aspergillus niger*, *Bacillus circulans*, *Streptomyces limosus*, *Clostridium thermosulfurogenes*, *Pseudomonas stutzeri*, *Klebsiella pneumoniae*, etc.). In analogy to CBD type I, II, III, and V, SBDs are rather rigid structures which are predominantly composed of ß-strands. Based on their interaction with maltose or cyclodextrin, usually two separate sugar-binding sites (1 and 2) can be distinguished in SBDs, which contain two or three exposed aromatic amino acids. The structure of site 1 is better conserved among the different SBDs than that of site 2, and has presumably a higher affinity for ligands than site 2. It contains two easily accessible Trp residues, which more or less keep their orientation upon ligand binding. Site 2 is much longer than site 1 and contains two or three Tyr residues which are located on a rather flexible loop of the SBD. Upon binding, site 2 undergoes significant structural changes which may allow the SBD to interact with starch in various orientations. In CGTases the SBD is part of a complex structure which comprises 5 separate domains. In these enzymes, site 1 is on the outside of the protein, whereas site 2 is more buried in the protein structure forming part of a channel leading to the catalytic site of the enzyme. Site-directed mutagenesis of aromatic amino acid residues belonging to both site 1 and 2 suggest that site 1 is the actual (raw) starch binding site whereas site 2 is involved in guiding glucan chains to

the catalytic site of the CGTase. It is not known whether site 2 also contributes to binding of starch granules.

Next to the SBDs, there are also proteins which only bind soluble α-glucans. An example of this is the maltodextrin-binding protein (MBP) from *Escherichia coli*. The amino acid residues that are involved in ligand binding are buried in the MBP structure. From the above, it can be seen that at least some the following factors may influence protein-carbohydrate interactions: (i) aromatic amino acids (often two or more) play a major role in these interactions; (ii) for binding crystalline structures these residues are positioned on an exposed face of the protein; (iii) for binding water-soluble molecules or amorphous structures these residues, or residues with a similar function are lining a groove, buried in the protein.

A number of enzymes involved in starch biosynthesis have been shown to associate with starch granules; one preferred subclass thereof according to the invention are the synthases. Reasons for this are the availability of several synthase genes and a mutant GBSS I (amf) potato plant (producing an amylose-free starch).

Example I: Determining factors involved in the granule-boundness of GBSS I.

WT, mutant and fusion proteins can be expressed in E. coli using a suitable expression system. Subsequently, the various enzymes can be purified, and their biochemical characteristics (activity, starch-binding) can be determined. Potato plants (both WT and amf background) can then be transformed with selected genes, based on the properties of their corresponding proteins. The forthcoming transgenic starch granules can then be analyzed for alterations in their size, morphology, and composition (including the fine structure of the polysaccharides if necessary). Thus, polymer production can be studied *in planta* with well-characterized proteins providing the essentials to understand why this polymer was made.

The amino acid sequence of GBSSI is determined in a manner known per se, for instance from the nucleotide sequence of an isolated GBSSI-encoding cDNA. Based on amino acid sequence alignments of various synthases a number of Trp or Tyr residues in the GBSSI sequnce can be selected as candidates for the interaction with starch granules. These (and combinations of these) can be replaced by site-directed mutagenesis. The mutant proteins are then be purified and analyzed for a reduced starch-binding capacity.

Potato (amf background) is then transformed with mutant GBSS I with reduced affinity to determine whether amylose biosynthesis can be restored to a similar extent as with WT GBSS I.

Based on the above experiments, aromatic amino acids can be introduced at appropriate positions in glgA from *Bacillus subtilis*, to study whether this enzyme can be made granule-bound. Potato plants (amf background) can then be transformed with mutant glgA ('s) to study whether amylose biosynthesis can be restored. In this way the starch-binding site of GBSS I can be mapped. Alternatively, the Tyr residues involved in binding could be replaced by Trp residues in order to tailor a GBSS I with improved starch-binding characteristics (as Trp residues are known to bind with higher affinity than Tyr residues).

Example II: Tailoring granule-boundness.

Granule-boundness may be tailored by fusing a relatively small SBD (100 amino acids), such as the SBD of the CGTase from Bacillus circulans, to a ("soluble") protein of interest.

This protein used can for instance be a soluble starch synthase, in order to provide amf potato plants that can be used as a model system for studying the potential of "artificial" granule-boundness *in planta* (i.e. to determine whether amylose biosynthesis in an amf background can be restored by expression of a fusion of the invention). Different fusions can be made. Next to synthases equipped with one SBD, also synthases with a two SBDs can be expressed, optionally with variations in in the linker peptides connecting synthase and SBD(s). The fusion proteins can also be expressed in *E. coli*, and subsequently purified to investigate their biochemical properties (starch-binding, activity). Potato plants (amf background) can be transformed with selected fusion proteins to study whether amylose biosynthesis can be restored to a similar extent as with WT GBSS I.

In addition, WT potato plants can be transformed with with a gene encoding a single SBD or a double SBD to determine the cpompetition between GBSS I and SBD(s) *in vivo*. This may also as an alternative way to make an amylose-free potato starch.

In these experiments, transformation of WT potato plants with (double) SBDs will also show whether GBSS I and SBD bind to similar parts (or structures) in the

starch granule (i.e whether GBSS I and SBD differ in their binding specificity). A different binding specificity would also provide the opportunity to target proteins to different parts of the starch granule, and also to use an inactive GBSS I (for instance obtained via genetical modification) as an alternative for a SBD in fusions. In addition, by making deletion mutants, different parts of GBSS I can be tested and used for their affinity for starch. (In this manner, alternative starch-binding domains could be obtained, with the same binding properties as GBSS I, but (much) of smaller size and and without catalytic activity.)

Example III: Enzyme fusions and possible applications.

Three possible enzymes that can be equipped with an SBD by expression as a fusion of the invention are the *Escherichia coli* glycogen branching enzyme GLGB, the potato kinase R1, and a glucose oxidase/dehydrogenase. The former two have already been expressed in potato without a SBD. By expression of these proteins as a fusion of the invention, their impact on starch structure and functionality can be increased.

Example III-1: Preparation of extra heavily branched amylopectin.

In a previous investigation by Applicant, the amount of branching in starch polymers was increased by introducing a heterologous branching enzyme in both WT and amf potato plants. The outcome of these experiments was as follows. (i) In an amf background an additional 25% of branching of the amylopectin was obtained. (ii) In a WT background evidence was obtained that next to the extra branching of amylopectin, amylose disappears as such. (Assuming that amylose is synthesized downstream of amylopectin, this indicates that Glgb is (partially) granule-bound.)

Additional branching comparwed to these reference tests might be achieved when glgB is specifically targeted to (and thus concentrated at) the granule surface by equipping the enzyme with a SBD, i.e. by expressing it *in planta* as a fusion of the invention. In this way, with a fusion of the invention, possibly a larger increase in branching can be obtained (i.e. compared to transforming with glgB per se), to provide improved freeze-thaw stability of starch solutions.

Example III-2: Phosphorylated starch.

Potato starch differs from many other starches in that it is more heavily phosphorylated. There is roughly one phosphate group to 700 Glc residues. Phosphorylation can occur at the C-6 (65%) or the C-3 position (35%) of Glc. The relatively large phosphate content gives potato tuber starch a number of unique properties such as the "peak viscosity" upon gelatinizing the granule, an anionic character, and possible anchors for derivatization.

Recently, an enzyme has been cloned which is presumably responsible for phosphorylation of starch. This kinase, further referred to as R1, cleaves ATP, and transfers a phosphate group to an unknown donor molecule. Phosphorylation of starch could be further increased by expressing a fusion of an SBD and R1 *in planta*, for instance in potato or cassava (heterologous expression).

Example III-3: Oxidized starch.

Starch contains a large collection of hydroxyl groups (-[CH2]-OH), but these are not very reactive. Next to these, each starch polymer contains an aldehyde group (-[CH]=O) at the reducing terminus, which is much more reactive. Because the starch polymers are such large molecules, the number of reactive groups is too small to be a meaningful target for derivatization. One way to increase the number of aldehyde or carboxyl (-[COH]=O) groups is by oxidation. As an alternative to chemical oxidation procedures, oxidases or dehydrogenases can be used, i.e. by expression as a fusion according to the invention. This could already lead to (increased) oxidation *in planta*, but also, and preferably, an oxidative enzyme fused to an SBD is incorporated in a starch granule during granule biogenesis, and subsequently these granules are incubated *in vitro* under conditions (temperature, pH, co-factors) suitable to provide oxidation of the starch.

Example IV: Constructs containing reporter genes.

Schematic representations of some non-limiting examples of constructs of the invention are shown in Figures 2B-2H. Instead of the luciferase gene shown in Figure 2, also another reporter gene such as (a sequence encoding a) beta-glucuronidase (GUS) can be used, or a sequence encoding the desired protein or polypeptide.

Briefly, the assembly of all constructs for potato transformation was started with the vector pBIN19^{PTT} (Fig. 2A), which already contained the tuber-specific GBSS I promoter, the amyloplast-targeting signal of potato GBSS I, and the NOS terminator sequence (for legend see figure). The starch-binding modules SBD and GBSS were obtained by standard PCR using the cyclodextrin glycosyltransferase of *Bacillus circulans* and potato granule-bound starch synthase I as a template, respectively. The luciferase template (pLUK07/LUC) was obtained from the North Carolina State University. PCRs were performed in such a way that the appropriate restriction sites were introduced in the genes of interest. The relevant restriction sites are indicated in Figure 2. An artificial linker sequence was designed, containing a BglII and an EcoRI restriction site at, respectively, the 5' and 3' end of the sequence. The amino acid sequence of the PT-rich linker peptide corresponds to "RSPTPTPTTPTPTPTTPTPTPSTE". The correctness of the constructs was confirmed by DNA sequencing. The constructs were introduced in both WT and amylose-free potato plants using standard *Agrobacterium*-mediated transformation procedures. The constructs provide the opportunity (i) to investigate whether SBD and GBSS bind the granule at a different location; (ii) to compare the affinity of SBD, SBD_2 and GBSS for starch during granule biosynthesis; (iii) to verify the concept of targeting foreign catalytic activities to the starch granule during biosynthesis.

SBD and double SBD were also cloned into a pTrcHisB vector (Invitrogen) in order to express both proteins in *Escherichia coli*. In these constructs, a 6xHIS tag was fused to the N-terminus of the proteins, which facilitated purification of these proteins from culture filtrates. The purified proteins were used for two purposes. (i) They were used in a standard rabbit immunisation procedure to obtain polyclonal antibodies. The antibodies recognized the SBD proteins in blotting experiments. (ii) They were used for *in vitro* starch granule binding assays. In a typical experiment SBD or SBD_2 are adsorbed on to the granule surface. The proteins can be desorbed with maltose. The concentration of maltose at which the proteins are released from the surface is indicative for the strength of binding.

The transgenic tubers can be subjected to Northern blot analysis using similar procedures as described in Salehuzzaman et al. (1999) Plant Cell Environment 22, 1311-1318. Starch granules can be isolated by grinding the tuber tissue in the presence of 0.5% (w/v) $Na_2S_2O_5$, followed by 3 washing/centrifugation steps with 30 mM

phosphate buffer pH7, and subsequently 3 washing/centrifugation steps with distilled water. The starch is then suspended in aceton, and air-dried. The starch can be subjected to Western blot analysis in essentially the same way as described in Salehuzzaman et al. [(1999) Plant Cell Environment 22, 1311-1318], using the polyclonal antibodies mentioned above. The structural features and/or physical properties of the starch can be further characterized by using (a selection of) the methods outlined below.

Example V: Constructs containing genes that can interact with starch.

In the constructs described in Example IV, the reporter gene may be replaced by a gene that can "interact with starch" as defined hereinbelow (e.g. by using in the protocol described in Example IV a nucleotide sequence encoding a gene that can interact with starch instead of the luciferase template.)

Examples of such genes may include, but are not limited to, a branching enzyme (e.g. from potato, pea, maize or *Escherichia coli*), an alpha-amylase (e.g from *Aspergillus oryzae* or *Bacillus licheniformis*), pullulanase (e.g. from *Klebsiella aerogenes*, isoamylase (e.g from *Pseudomonas amyloderamosa*), amylomaltase (e.g. from *Bacillus*), sucrase (e.g. from *Leuconostoc mesenteroides* or *Streptococcus mutans*, a potato kinase (e.g. for adding phosphate groups to starch), an oxidase (e.g. glucose oxidasefrom *Aspergillus niger* or a dehydrogenase (e.g. glucose dehydrogenasefrom *Acinetobacter calcoaceticus* or *gluconobacter*).

The constructs thus obtained can be transformed into a starch-producing plant, such as potato, e.g. using Agrobacterium as described in Example IV.

The transformed potato plants thus obtained can be used to produce tubers that contain starch (granules) with altered properties compared to the starch (granule) natively produced by the potato plant.

CLAIMS

1. Method for expressing a desired protein or polypeptide in a plant, in which the protein or polypeptide is expressed as a fusion with at least one starch binding domain.

2. Method according to claim 1, comprising the steps of:

a) providing a genetic construct comprising at least one nucleotide sequence encoding the desired protein or polypeptide combined with at least one nucleotide sequence encoding a starch binding domain, so that the construct encodes a fusion of the desired protein/polypeptide and the at least one starch binding domain;

b) transforming a plant with said genetic construct;

c) expressing said genetic construct in the plant.

3. Method according to claim 1 or 2, in which the plant is a plant that contains or produces starch or starch granules in at least one of its parts, including its seeds, leaves, roots (including tuburous roots), tubers, stems, stalks, fruits, grains or flowers (in particular the honey-producing parts thereof).

4. Method according any of the preceding claims, in which the plant is chosen from potato, sweet potato, cassava, pea, taro, sago, yam, banana, and/or cereals such as rice, maize, wheat and barley.

5. Method according to any of the preceding claims, in which the protein or polypeptide is heterologous with respect to the plant in which the fusion is expressed.

6. Method according any of the preceding claims, in which the protein or polypeptide is an enzyme.

7. Method according to claim 6, in which the enzyme is an enzyme that can interact with starch or starch granules, in particular an enzyme that can convert, modify, alter, degrade or otherwise influence the starch, the starch granule or the structure or interactions thereof.

8. Method according to any of claims 1-5, in which the desired protein or polypeptide is a receptor such as an estrogen receptor or a plant hormone receptor, or a structural protein such as a protein "zipper".

9. Fusion of at least one desired protein or polypeptide and at least one starch binding domain, as expressed in and/or present in a plant or in any part of a plant, including its seeds, leaves, roots (including tuburous roots), tubers, stems, stalks, fruits, grains or flowers (in particular the honey-producing parts thereof).

10. Genetic construct suitable for transforming a plant, comprising at least one nucleotide sequence encoding a desired protein or polypeptide combined with at least one nucleotide sequence encoding a starch binding domain, so that the construct encodes a fusion of the desired protein/polypeptide and the at least one starch binding domain.

11. Method for providing a plant that expresses a fusion according to claim 9, comprising at least one step of:

a) transforming plant with a genetic construct according to claim 10, such that said genetic construct is expressed in the plant or at least in part thereof;

and optionally further comprising at least one step of:

b) providing descendants and/or further generations of the thus transformed plant, for instance via sexual or asexual multiplication, including crossing and/or other breeding techniques.

12. Method according to claim 11, in which the plant is a plant that contains or produces starch or starch granules in at least one of its parts, including its seeds, leaves, roots (including tuburous roots), tubers, stems, stalks, fruits, grains or flowers (in particular the honey-producing parts thereof).

13. Plant, transformed with a genetic construct according to claim 9, or a descendant of such a plant.

14. Plant that expresses a fusion according to claim 9, obtainable by the method of claim 10.

15. Seeds, tubers, seedlings or other cultivating material of a plant according to claim 13 or 14.

16. Method for providing a protein or polypeptide with affinity for starch and/or starch granules, and/or for increasing the affinity of a protein or polypeptide for starch and/or starch granules, comprising expressing the protein or polypeptide in a plant as a fusion with at least one starch binding domain.

17. Method according to claim 16, comprising the steps of:

a) combining a nucleotide sequence encoding the protein or polypeptide with at least one nucleotide sequence encoding a starch binding domain, so as to provide a genetic construct encoding a fusion of the protein or polypeptide and the at least one starch binding domain;
b) transforming a plant with said genetic construct;
c) expressing said genetic construct in the plant.

18. Method for producing a complex of at least one protein or polypeptide and a starch granule, comprising at least one step of:

a) expressing the protein or polypeptide as a fusion with at least one starch binding domain, in a plant that contains or forms starch granules;

and optionally comprising a further step of:

b) isolating the protein or polypeptide from the plant or any part thereof as a complex of the fusion and the starch granule.

19. Complex, comprising a fusion of a protein or polypeptide fused to at least one starch binding domain, associated with a starch granule.

20. Method for providing a plant that can produce a complex according to claim 19, comprising at least the step of:

a) transforming a starch granule producing plant with a genetic construct according to claim 10, such that said genetic construct is expressed in the plant or at least part thereof;

and optionally further comprising at least a further step of:

b) providing descendants and/or further generations of the thus transformed plant, for instance via sexual or asexual multiplication, including crossing and/or other breeding techniques.

21. Plant that produces a complex according to claim 18, and that is obtainable via the method of claim 20, or any descendant thereof.

22. Seeds, tubers, seedlings or other cultivating material of a plant according to claim 21.

23. Method for providing a plant that contains or produces a modified starch and/or modified starch granules, comprising at least one step of:

a) transforming a starch producing plant, in particular a starch granules producing plant, with a genetic construct comprising at least one nucleotide sequence encoding an enzyme that can interact with starch and/or starch granules and at least one nucleotide sequence encoding a starch binding domain, such that said genetic construct is expressed in the plant or at least part thereof;

and optionally further comprising at least a further step of:

b) providing descendants and/or further generations of the thus transformed plant, for instance via sexual or asexual multiplication, including crossing and/or other breeding techniques.

24. Plant that produces modified starch or starch granules, and that is obtainable via the method of claim 23, or any descendant thereof.

25. Seeds, tubers, seedlings or other cultivating material of a plant according to claim 24.

26. Method for producing a modified starch and/or modified starch granules, comprising at least one step of:

a) cultivating a transformed plant that produces a modified starch and/or modified starch granules according to claim 24, or a descendant thereof;

and optionally comprises at least one further step of:

b) isolating the modified starch or starch granules from the transformed plant or any part thereof, including its seeds, leaves, roots (including tuburous roots), tubers, stems, stalks, fruits, grains or flowers (in particular the honey-producing parts thereof).

27. Method for producing a modified starch and/or modified starch granules, comprising at least one step of:

a) cultivating a transformed plant that expresses a fusion of at least one starch binding domain and at least one enzyme that can interact with starch (granules), in which the starch (granule) and the fusion preferably form a complex;

b) harvesting the plant and/or any part of said plant that contains starch (granules) and the fusion, such as the seeds, leaves, roots (including tuburous roots), tubers, stems, stalks, fruits, grains or flowers (in particular the honey-producing parts thereof);

c) subjecting the plant and/or plant material, or any fraction or preparation obtained therefrom that contains the starch (granule) and the fusion, to conditions such that the enzyme can interact with the starch (granule) to provide modified starch or starch granules;

and optionally comprises at least one further step of:

d) isolating the modified starch or starch granules thus obtained.

28. Modified starch or starch granules, obtained via the method of claim 26, obtained from a plant according to claim 24 or any part thereof, or obtained via the method of claim 27.

29. Method for providing a plant that contains or produces a modified starch and/or modified starch granules, comprising at least one step of:

a) transforming a starch producing plant, in particular a starch granules producing plant, with a genetic construct comprising at least one nucleotide sequence encoding a starch binding domain, such that said genetic construct is expressed in the plant or at least part thereof;

and optionally further comprising at least one step of:

b) providing descendants and/or further generations of the thus transformed plant, for instance via sexual or asexual multiplication, including crossing and/or other breeding techniques.

30. Plant that produces modified starch or starch granules, obtainable via the method of claim 29, or any descendant thereof.

31. Seeds, tubers, seedlings or other cultivating material of a plant according to claim 30.

32. Method for producing a modified starch and/or modified starch granules, comprising at least one step of:

a) cultivating a transformed plant that produces a modified starch and/or modified starch granules according to claim 27, or a descendant thereof;

and optionally further comprises at least one step of:

b) isolating the modified starch or starch granules from the transformed plant or from any part thereof, including its seeds, leaves, roots (including tuburous roots), tubers, stems, stalks, fruits, grains or flowers (in particular the honey-producing parts thereof)

33. Modified starch or starch granules, obtained via the method of claim 32, or obtained from a plant according to claim 30 or any part thereof.

34. Modified starch according to claim 33, being an essentially amylose-free starch or starch granule.

35. Genetic construct suitable for transforming a plant, comprising at least one nucleotide sequence encoding a starch binding domain.

35. Bacterium, virus or other organism suitable for transforming a plant, containing a genetic construct according to claim 10 or 35, and preferably capable of transferring said construct into a plant.

Fig 1

Alignment of starch-binding domains

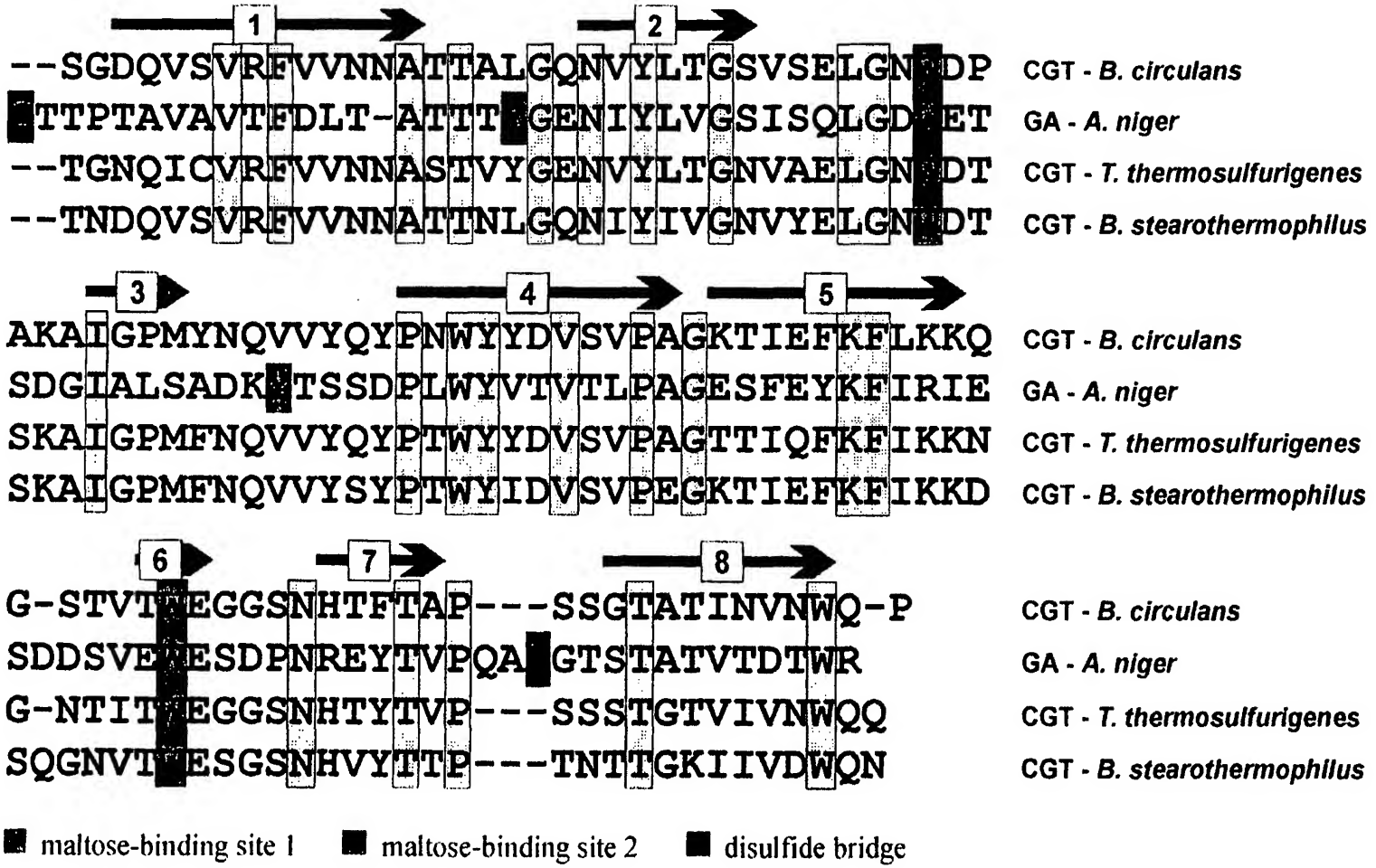
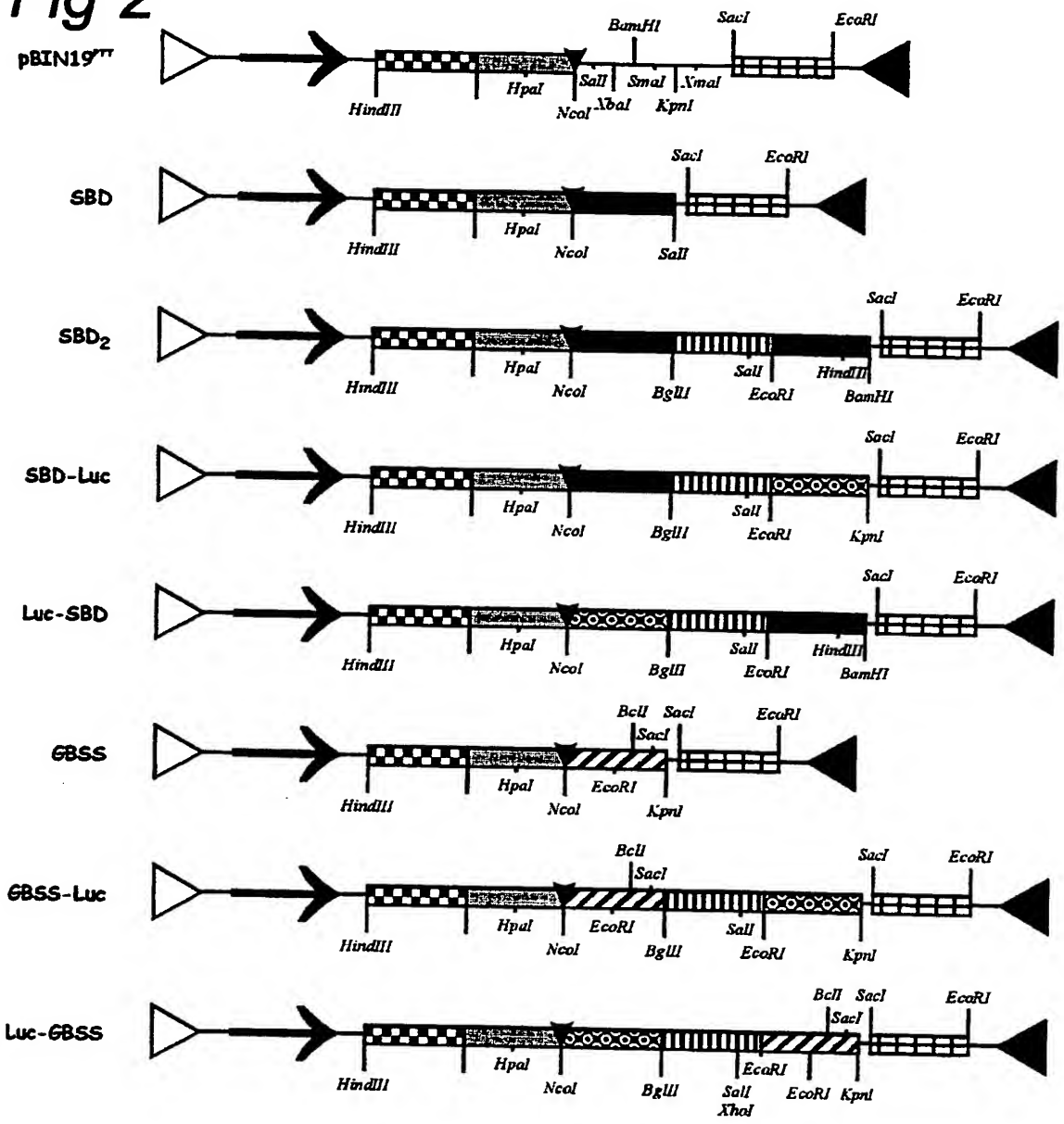


Fig 2


pBIN19^{TT}
BamHI
SacI
EcoRI
HindIII
HpaI
SalI
SmaI
XmaI
NcoI
XbaI
KpnI
SBD
SacI
EcoRI
HindIII
HpaI
NcoI
SalI
SBD$_2$
SacI
EcoRI
HindIII
HpaI
NcoI
BglII
SalI
EcoRI
HindIII
BamHI
SBD-Luc
SacI
EcoRI
HindIII
HpaI
NcoI
BglII
SalI
EcoRI
KpnI
Luc-SBD
SacI
EcoRI
HindIII
HpaI
NcoI
BglII
SalI
HindIII
EcoRI
BamHI
GBSS
BclI
SacI
EcoRI
HindIII
HpaI
NcoI
EcoRI
KpnI
GBSS-Luc
BclI
SacI
SacI
EcoRI
HindIII
HpaI
NcoI
EcoRI
BglII
SalI
EcoRI
KpnI
Luc-GBSS
BclI
SacI
SacI
EcoRI
HindIII
HpaI
NcoI
BglII
EcoRI
SalI
XhoI
EcoRI
KpnI
left border
right border
NPT II
cleavage site transit peptide
potato GBSS I promoter (tuber-specific)
potato GBSS I transit peptide
SBD
PT-rich linker
GBSS I
luciferase
NOS terminator

PATENT COOPERATION TREATY

REN

ONTVANGEN

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

08 OKT 2001

SVP bezorgdelen
Relevant
NRF2 11-12-2001

AMERSFOORT PCT

To:

VEREENIGDE
Nieuwe Parklaan 97
P.O.Box 87930
2508 DH Den Haag
PAYS-BAS

Kopie voor | TERMIJN
02 OKT 2001
Antwoord Voorl. oel. | bericht gezonden aan dd.
MAP P57575PC00

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	27.09.2001
Applicant's or agent's file reference BO 42696	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL00/00406	13/06/2000	11/06/1999

Applicant
LANDBOUWUNIVERSITEIT WAGENINGEN et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Faux, K Tel.+49 89 2399-8062

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
BO 42696		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL00/00406	13/06/2000	11/06/1999
International Patent Classification (IPC) or national classification and IPC C12N15/54		
Applicant LANDBOUWUNIVERSITEIT WAGENINGEN et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
10/01/2001	27.09.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Kania, T Telephone No. +49 89 2399 7703

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL00/00406

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-40 as originally filed

Claims, No.:

1-36 as originally filed

Drawings, sheets:

1/2-2/2 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-36
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-36
Industrial applicability (IA)	Yes:	Claims	1-36
	No:	Claims	

2. Citations and explanations
   see separate sheet

The present IPER refers to the documents cited in the search report.
The document numbering (D1-D6) corresponds to their order of citation in the search report.

Section V (novelty and inventive step, Art. 33 PCT)

1. The present application discloses the use of starch binding domains (SBDs) for the expression as fusions with proteins of interest in transgenic plants. The proteins of interest thereby achieve an affinity to starch or starch granules, which renders them easily purifyable from the plants. Further, proteins that interact with starch may be fused to an SBD. Thereby modified starch can be produced.

2. Cited document D4 discloses methods and products for the encapsulation of proteins into the starch matrix of transgenic plants. The proteins of interest are expressed as fusions with a "starch encapsulation region", which is a diffferent expression for the SBDs used in the present application. In the examples, SBDs from starch-modifying enzymes, like starch synthases, are used. The methods are useful for the purification of proteins of interest from transgenic plants, as well as for the protection of these proteins from the digestive action of proteases when used as animal food.

3. Document D4 thus comprises all features of the present application as claimed in claims 1-6, 8-22, 24-26, 28, 36.

4. Claims 29-35 relate to constructs comprising at least a SBD and their use in methods for providing plants with modified starch and/or starch granules, as well as to the modified starch, preferably amylose-free starch.
Cited document D3 discloses transgenic plants comprising essentially amylose-free starch, that were produced via the recombinant expression of a granule-bound starch synthase. The enzyme comprises a starch binding domain.

Thus, claims 1-6, 8-22, 24-26, 28-36 are considered as **not novel**.

5. A feature that distinguishes the present application from D4 is the selection of starch-modifying enzymes as proteins of interest, and the use of the resulting fusions for the modification of the fine structure of starch and starch granules.

Claims 7, 23, and 27, however, relate to "enzymes that can interact with starch or starch granules", and, as formulated in claim 7, that "influence the starch, starch granule or the structure or interaction thereof".
It is to be noted, that any enzyme expressed in the starch granule can be considered to "alter the starch" or " modify its structure".

Thus, claims 7, 23, 27 are **not novel**.

Further, in the light of the prior art disclosing methods for the modification of starch fine structure in transgenic plants by recombinant expression of starch-modifying enzymes (see e.g. D2, D3), it appears to be an obvious step to replace the proteins of interest used in D4 by a starch-modifying enzyme.
Thus, claims 7, 23, 27, if rendered novel, would not be considered inventive.

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
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International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL00/00406	13/06/2000	11/06/1999
International Patent Classification (IPC) or national classification and IPC C12N15/54		
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Date of submission of the demand	Date of completion of this report
10/01/2001	27.09.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Kania, T Telephone No. +49 89 2399 7703

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL00/00406

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	No:	Claims	1-36
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-36
Industrial applicability (IA)	Yes:	Claims	1-36
	No:	Claims	

2. Citations and explanations
   see separate sheet

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